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Effect of Low Energy Argon Laser and  
Dihematoporphyrin Ether on the Growth,  
Viability, and Catecholamine Production  
of C1300 Murine Neuroblastoma Cells in Vitro

by  
David William Rogers

Submitted to  
Rochester Institute of Technology  
in Partial Fulfillment of the Requirements  
for the Degree of

Master of Science

Department of Clinical Sciences  
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1988

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## ABSTRACT

### Effect of Low Energy Argon Laser and Dihematoporphyrin Ether on the Growth, Viability, and Catecholamine Production of C-1300 Neuroblastoma Cells in Tissue Culture

Low power laser induced fluorescence of dihematoporphyrin ether (DHE, photofrin 2) is used to detect cancer. The goal was to determine the effect of low power argon laser light and DHE on C-1300 murine neuroblastoma cells (MNB, NB41A3). The study groups were as follows: Control, untreated cells (C), DHE only at concentrations of 1 (D1) and 5 (D5)  $\mu\text{g/ml}$ , Argon laser light only at exposure times of 1 minute ( $1.8 \text{ J/cm}^2$ , L1) and 5 minutes ( $9.0 \text{ J/cm}^2$ , L5), combined treatment of Light at 1, 5 minutes, and DHE at 1, 5  $\mu\text{g/ml}$ .

Nine replicates were performed following incubation of cells in either 1.0  $\mu\text{g}$  DHE/ml or 5  $\mu\text{g}$  DHE/ml for two hours. The Cooper Lasersonics lexel model 150 argon laser was used.

Cell growth was measured by counting cells, and cell viability by trypan blue exclusion was determined post treatment at 1, 24, 96, and 144 hours. Catecholamine analysis of cell supernatant was performed by Amersham Radiocenzymatic Cat-A-Kit<sup>R</sup> assay. The results were: cell numbers decreased immediately and remained low for 9 days in the groups treated with Light for 1 and 5 minutes and 5.0  $\mu$ g DHE/ml, and in Light 5 minutes with 1.0  $\mu$ g DHE/ml group as compared with all other treatment groups. Cell viability followed the same trend with the L5 group, L1 at 5  $\mu$ g DHE/ml, and L5 group at 1  $\mu$ g DHE/ml as compared with group C. Cell morphology was altered in these groups. Cells changed shape becoming rounded, contained vacuoles, and pyknotic inclusions.

It was concluded that light alone with 5 minutes exposure kills cells, but light alone with 1 minute exposure has no effect on C1300 MNB cells. However, cells were damaged and killed, affected in groups L1D5, L5D1, and L5D5. Catecholamine concentration was below the detection limit of the assay for most samples. Therefore, no interpretation can be made about using catecholamines as a marker of cell status.

Low energy argon laser light at the power density of  $30 \text{ mW/cm}^2$  and DHE concentrations of 1 and  $5 \mu\text{g/ml}$  that is used for detection of cancer causes changes in cell morphology, number, and viability in this in vitro model.

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## ABBREVIATIONS

DHE	Dihematoporphyrin ether, Photofrin 2
HPD	Hematoporphyrin derivative
MNB	Murine Neuroblastoma Cells
PDT	Photodynamic Therapy
LIF	Laser-induced Fluorescence
mW	milliwatts
cm	centimeter
J	joules
$\mu$ g	micrograms
m	meter
ANOVA	analysis of variance
pg	picogram

## INTRODUCTION

Low power, low energy argon laser induced fluorescence (LIF) of dihematoporphyrin ether (DHE) is used to detect cancer (1). Photographic methods using filtered flashes can detect DHE fluorescence in surface lesions with concentrations of DHE as low as 50 nanograms (2). Endoscopic detection of cancer inside body cavities requires the use of laser light.

It is known that high energy laser light and the photosensitizers DHE or hematoporphyrin derivative (HPD) kill cancer cells both in vivo (3,4) and in vitro (5,6,7, 8). The in vitro studies involved varying laser energy densities (joules) and photosensitizer concentrations to determine maximum killing of cells in monolayers in tissue culture.

Current techniques of endoscopic detection of cancer use 10 to 50 milliwatts (mW) per square centimeter of laser power with the lowest possible tissue concentration of photosensitizer, about 1 to 10 micrograms ( $\mu$ g) per gram (9).

Laser light alone affects cell metabolism. Low energy argon laser has cytotoxic effects on Chinese hamster cells in culture (10). Low energy helium cadmium laser light has been shown to possess marked anti-inflammatory action (11) and prevents mitosis in mouse eggs (12). Helium neon laser light affects cell membranes (13) and growth (14). Effects on cell metabolism and structure is dependent on the wavelength, power density, and energy density of laser light. Research is needed to determine the effect of low power ( $<400 \text{ mW/cm}^2$ ), low energy ( $<20 \text{ Joules/cm}^2$ ) argon laser on cancer cells, with and without photosensitizers. Low energy laser light is defined as that level at which there is no thermal effect, only photodynamic effect.

Neuroblastoma is the most common extracranial malignant solid tumor in children (15,16). It occurs in 500 children yearly in the United States (17). Neuroblastoma is an extensive, locally invasive cancer (15) found in the peritoneal cavity and bone marrow. LIF detection and photodynamic therapy (PDT) are promising new modalities for identifying and treating neuroblastoma. Makino (3) has shown that PDT is effective in producing 50% kill in tumor in highly malignant neuroblastoma xenographs and C1300 murine neuroblastoma (MNB).

Neuroblastoma is detected and diagnosed by assaying for elevated levels of catecholamines, specifically dopamine, in serum (18), plasma (19), and tissue (20,21). This specific tumor marker for neuroblastoma may allow quantitation of tissue-tumor cell destruction when using cancer killing therapies like PDT or cancer detection with LIF of DHE.

LIF of DHE may be used in locating neuroblastoma in vivo, both at the surgical site and in bone marrow. It is necessary to understand the effect that low energy argon laser light and photosensitizers may have on metabolism of neuroblastoma cells before LIF of DHE is used as a cancer detection method.

In this study, C1300 MNB in tissue culture was used as a model system to study the effects of low energy argon laser and the photosensitizer, DHE on cell growth as an indicator of cell metabolism and reproduction; cell viability by trypan blue dye exclusion; and explore the changes in catecholamine metabolism by measuring catecholamine content of cell medium by the differential radioenzymatic assay.

The clinical significance of this study is the possibility of being able to infer the amount of tissue damage to the tumor and surrounding tissue, predict any increased proliferation of tumor, and to quantify change in metabolism of tumor cells by measuring serum catecholamine levels following LIF of DHE for detection of neuroblastoma.



## BACKGROUND

### **Photodynamic Therapy (PDT) and Dihematoporphyrin Ether (DHE)**

"Photodynamic therapy is a new and exciting treatment for many forms of localized malignant disease" (22). PDT can cause a complete response; that is, kill all malignant tissue in 60% to 70% of the cases suitable for this type of treatment (23).

The treatment protocol consists of first injecting the photosensitizer intravenously. When the photosensitizer, dihematoporphyrin ether (DHE), is used in a dosage of 1-5 mg/kg body weight of the patient, DHE is taken up by all cells. It is retained longer by the liver, spleen, skin, intestinal mucosa, and malignant tumor tissue than it is in other normal body tissues.

After a period of 24 to 48 hours, when normal tissue contains a lower concentration of DHE than does malignant tissue, the involved area is irradiated with light. Most investigators (23,24,25) use red laser light produced by the argon-dye or gold metal vapor lasers. The suggested power density is from 14 to 400 mW/cm<sup>2</sup> and

the energy density used varies between 25 and 200 joules/cm<sup>2</sup>, depending on the volume and location of the malignant lesion (23).

Photodynamic therapy is a promising modality for treating tumors that are resistant to chemotherapy and ionizing radiation therapy and also for primary local malignant disease, such as neuroblastoma (58).

#### **Cancer Detection by DHE Fluorescence**

Laser induced fluorescence has proven useful as a diagnostic technique in identifying occult bladder (26) and bronchial tumor as small as 1 mm<sup>2</sup> (27). Endoscopic detection of malignant disease requires intense laser light.

DHE fluorescence in surface lesions and tumor tissue exposed intra-operatively may be detected when activated with laser irradiation (1) and by filtered flash photography (2).

Argon (488,514 nm), krypton (405,413 nm), nitrogen (337 nm), and helium cadmium (442 nm) laser light is known to stimulate red (630 nm) fluorescence of hemato-



porphyrin derivative (HPD) and DHE accumulated in tumor cells in vitro and in vivo (1,5,23,28,29,30,31).

For diagnosis, laser power densities of 10 to 50 mW/cm<sup>2</sup> seem to be effective in causing fluorescence sufficient to identify concentrations of 1 µg DHE/ml in tissue.

#### **The Photosensitizer - Dihematoporphyrin Ether**

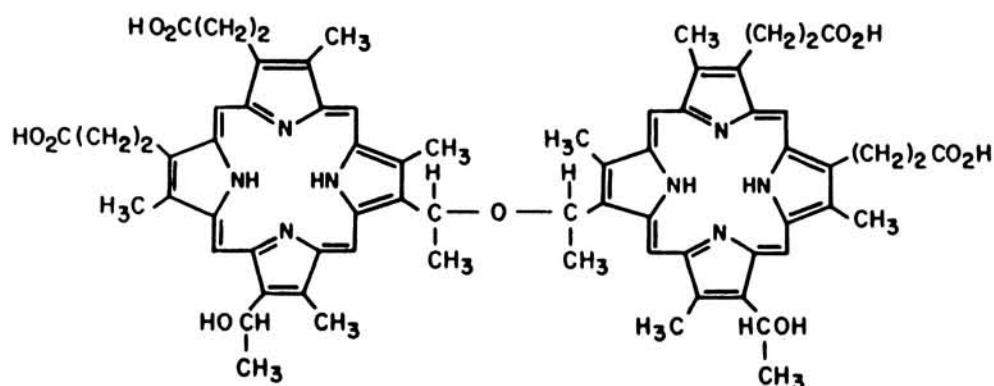
Dihematoporphyrin ether is a somewhat purified component of hematoporphyrin derivative (32). Hematoporphyrin derivative was first prepared by Lipson (32). Dihematoporphyrin ether is available as Photofrin 2<sup>R</sup>.

Hematoporphyrin derivative is derived from hemoglobin by treatment with acetic and sulphuric acids to modify the side chains of the porphyrin group. There are seven distinct components to hematoporphyrin derivative (HPD). The formula for HPD is C<sub>34</sub>H<sub>38</sub>O<sub>6</sub>N<sub>4</sub>. Molecular weight is 588.7. It is excited at 405 to 630 nm. The wavelength maximum is 405 nm. It fluoresces between 600 and 700 nm. The fluorescence maximum is 630, with a second maximum at 690 nm. It forms aggregates in aqueous solution. It is lypophillic. Its uptake and retention,

cancer cells to normal cells, is 10:1. Standard dose is 1 to 5 mg of HPD/kg bodyweight. Its retention time is 24 to 72 hours. A side effect of this treatment is photosensitivity, especially of skin and eyes. Elimination pathway is the liver and the gastrointestinal tract. Dihematoporphyrin ether, the so called active component of HPD, has a molecular weight of approximately 627 (32). The structure appears in Figure 1. The absorption emission spectrum (Figure 2) of DHE is similar to HPD.

Photosensitization is a photodynamic action involving light, oxygen, and the photochemical, DHE. There are two types of reactions. Type 1 reaction is a direct photosensitizer interaction which produces proton or electron donors. In the Type 2 reaction, the photosensitizer transfers excitation energy to the oxygen ground state forming singlet oxygen (33,34).

Fluorescence occurs when light activated DHE singlet state is formed and decays to the ground state. The DHE singlet state can decay to the triplet state to release light as phosphorescence or react with oxygen to form singlet oxygen (33). The reactive singlet oxygen tears apart proteins, oxidizes lipids in biological membranes (31), and interferes with cytochrome c oxidase in the mitochondria (35).



Bis - 1 - [ 8 - ( 1 - hydroxyethyl ) deuteroporphyrin - 3 - yl ] ethyl ether

Figure 1. Structure of HPD-active component.

Dougherty, Potter, Weishaupt (32)

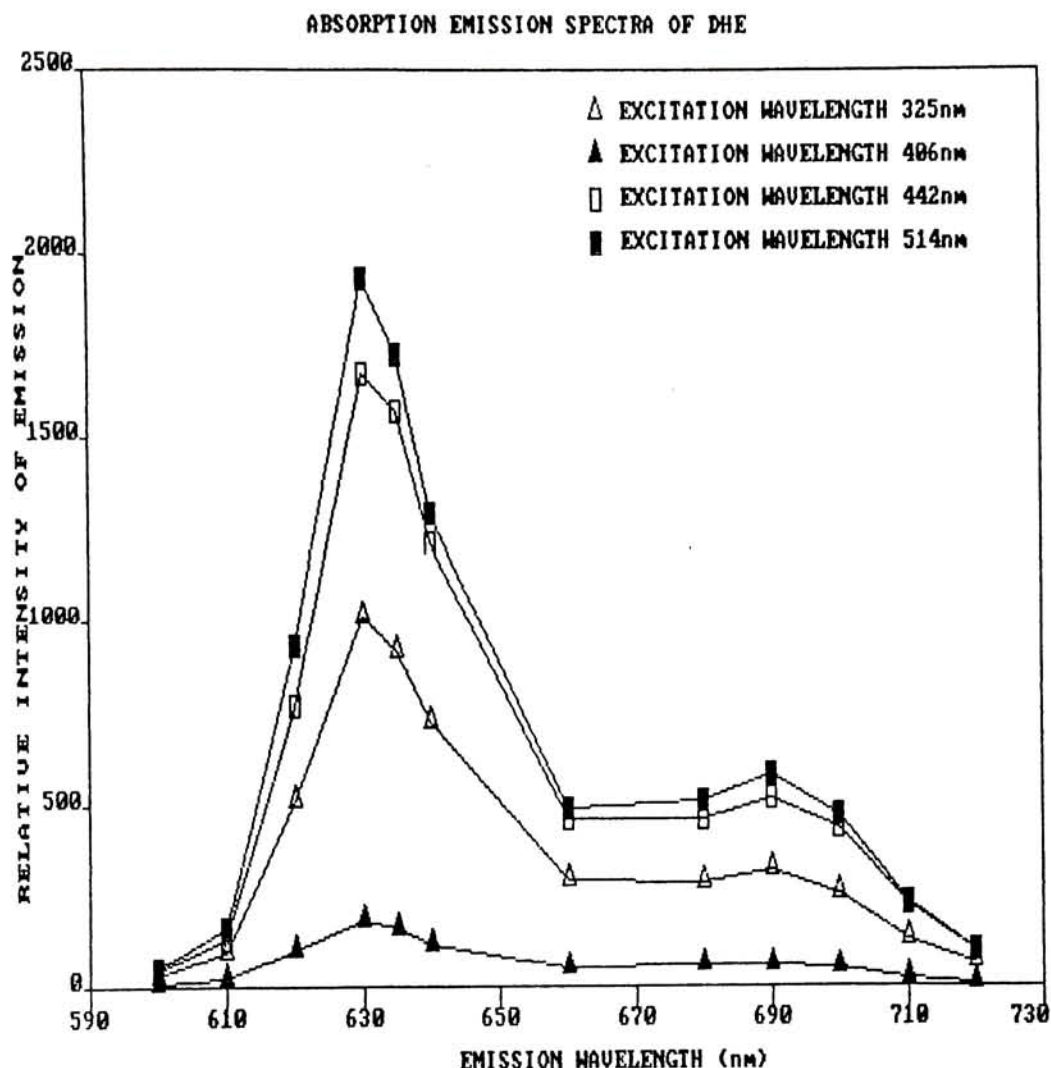


FIGURE 2. Absorption Emission spectrum of DHE.

Absorption 590-730nm, Emission - 630nm.

Obtained on Perkin Elmer LS-5 spectrofluorimeter. 50  $\mu$ g DHE dissolved in 95% Ethanol.

Serum proteins are responsible for porphyrin transport in the bloodstream and the endocellular concentration of hematoporphyrin (Hp) and related porphyrins (36). A higher concentration of DHE/HPD is needed to photosensitize cells in tissue culture if the medium contains serum.

Uptake rate and cellular deposition of DHE and HPD in tumor cells in vivo and in vitro have been characterized by many investigators (26,29,31,37,38). Most cell lines take up DHE in 1 to 24 hours at doses ranging from 1 to 25  $\mu\text{g/ml}$  (37). Berns (37) has shown uptake into cytoplasm and nuclear membranes peaked at 24 hours with concentrations of 25  $\mu\text{g/ml}$ . Berns (37) achieved similar photodynamic kill of Chinese hamster ovary cells incubated for one hour with 25  $\mu\text{g}$  HPD/ml plus 1% serum and when incubated for 12 hours with 25  $\mu\text{g}$  HPD/ml plus 5% serum. Power levels were 5-100  $\text{mW/cm}^2$  of either argon dye (630 nm) or gold vapor laser (628 nm).

DHE binds nonspecifically to serum protein. Increased cytotoxic effect of DHE occurs with shorter incubation time, if serum is omitted from tissue culture medium (39). This information helped determine the incubation time of cells with DHE in the experimental design of the work reported here.



Today, experimental studies in humans, animal, and cell culture tumor models (38) are underway with DHE and with other photosensitizers such as Cryptocyanines, Nile Blue A, Dequalinium, Chloroaluminum phthalocyanine tetrasulfate, chlorins and purpurins.

### **Photobiology and Photochemistry**

Phototherapy is a common clinical modality. Some uses are: 1) to increase pigmentation by increasing melanin production and numbers of melanocytes; 2) to reduce tissue bilirubin concentration by using blue (454 nm) light; and 3) to alter cortisol levels, thus circadian rhythm, by controlling light exposure via the day-night cycle.

Laser technology has stimulated basic and clinical research in PDT, biostimulation, and biosuppression. Laser-induced fluorescent detection of DHE in malignant tissue involves the use of low energy laser light. Each wavelength or color of light has specific absorption energy levels, and this determines the selective absorption by the different photochemicals.

Photodynamic therapy (PDT) of cancer cells in culture had been performed by various investigators. Most PDT involves the use of 630 nm light at laser powers of 100 milliwatts (mW) to 1 watt (W) and energy densities of 100 to 300 J/cm<sup>2</sup> (28). Light transmission through tissue is maximized at red (630 nm) wavelengths.

Anderson has shown that violet laser light (405 nm) is more effective at killing cells in culture at low power densities (i.e., 20-40 mW/cm<sup>2</sup>) and low doses of HPD (1-25 µg/ml HPD) than red light. Violet light causes quicker kill than 630 nm red light (30), because DHE absorbs light more efficiently at 400-520 nm.

Low energy and low power laser light has been shown to stimulate and/or inhibit cell growth (41,42,43). Low light inhibits causes changes in light sensitive amino acids and proteins. Argon (514 nm) laser light alone at energy densities of 100 to 400 J/cm<sup>2</sup> has cytostatic and cytotoxic effects (42).

Mester (43) reviewed laser light effects on 15 biological systems and reported that low energy 1 J/cm<sup>2</sup> of ruby laser light stimulated phagocytosis in leukocytes and increased mitotic index in Erhlich acites tumors but

2 to 4 J/cm<sup>2</sup> inhibited leukocytic phago-cytosis. He used HeNe (50 mW, 630 nm) and Argon (100 mW, 400 nm) at energy density of 4 J/cm<sup>2</sup>, two times a week on the total wound surface and this speeded healing of ulcers. Kubasova (13) showed that 1 J/cm<sup>2</sup> of the HeNe laser light applied once every 24 hours for four days changed the surfaces of exposed cells. Cells showed pits. This information is important in support regarding changes in cellular morphology and growth characteristics of low energy argon laser light radiation (488, 514 nm) without DHE in the cells.

### **Neuroblastoma**

Neuroblastoma is a hormone and amine secreting tumor derived from sympatheticoblast melanocytes and, therefore, from embryonic neural crest cells (44). Neuroblastoma and pheochromocytomas share with neural tissue the capacity to synthesize and release biogenic amines: histamine, 5-hydroxytryptamine (serotonin, 5-HT), dopamine, and, in some tumors, norepinephrine and epinephrine (44).

Catecholamines, specifically dopamine (3,4-dihydroxyphenylethylamine) and the breakdown product of dopamine, homovanillic acid (HVA), are indicators of neuroblastoma (18) and, rarely, of pheochromocytoma (45).



Various methods are used to measure catecholamines for the diagnosis of neuroblastoma.

Reynolds reported the use of catecholamine fluorescence and morphology of cells isolated from human tissue and grown in tissue culture to differentiate neuroblastoma from ganglioneuroma and pheochromocytoma (46). Proye reported that pheochromocytomas do secrete dopamine (47). Techniques of catecholamine fluorescent staining and observation of the morphology of cells grown in tissue culture are more specific for diagnosing neuroblastoma than is measurement of urinary VMA and catecholamine (46). Helson used a formaldehyde induced fluorescent method for the detection of intracellular catecholamines in neuroblastoma cells in human bone marrow aspirates and cultured cells from human and murine sources (C1300 tumor)(48).

C1300 Murine Neuroblastoma (MNB) has been identified as a model of human disease (45,50,51,52). These cells contain and secrete catecholamines (45,46,49,51). Pons (51) reported levels of catecholamines from biological specimens from control and C1300 MNB tumor implanted animals. Catecholamines were assayed by using HPLC and an electrochemical detector. In primary tumors, dopamine and norepinephrine were detected, but there was no epinephrine. Pons (51) reported the following levels:

**TABLE 1**  
Catecholamine Levels

	Tumor X value (ng/g) n=10	Serum (ng/ml)	
		Control	Tumor
DA	28 $\pm$ 14	0.4 $\pm$ 0.4	21 $\pm$ 2.3
NE	207 $\pm$ 40	19.3 $\pm$ 5.1	39 $\pm$ 7.7
E	ND	23.2 $\pm$ 13.9	12.8 $\pm$ 4.7

These levels are 1,000 times greater than is expected in monolayer cell cultures.

Catecholamine levels in plasma decrease following surgical excision of tumor and may increase with recurrence (53). Plasma and 24-hour urinary estimates are the best sample choices since plasma levels may be elevated in the absence of free urinary catecholamines; tumors excrete catecholamines inconsistently and urinary excretion follows a diurnal pattern (53).

### Catecholamines

The catecholamine neurotransmitters are noradrenalin, adrenalin, and dopamine (3,4-dehydroxyphenylethylamine). Catecholamines are synthesized from L-tyrosine through a series of enzymatic reactions that

form dopamine, noradrenalin, and adrenalin. In the parts of the nervous system that release dopamine as a neurotransmitter, no further metabolism occurs and dopamine is stored in vesicles in the presomatic nerve terminal. Catabolism of catecholamines occurs by amine degradation by the enzyme amine oxidase. Catecholmethyltransferase and aldehydedehydrogenase degradation occurs in all tissues including nerves, liver, intestine, and kidney. Figure 3 shows the synthetic and degradation pathways of catecholamines.

Biosynthesis of catecholamines is rate limited by the level and activity of tyrosine hydroxylase (56). This enzyme is oxygen dependent and kinetic properties suggest oxygen availability may limit synthesis of catecholamines (55). Tyrosine hydroxylase is inhibited by dopamine and norepinephrine (54).

Dopamine is converted to norepinephrine by the action of dopamine- $\beta$ -hydroxylase. This is a mixed function oxidase requiring oxygen and a cosubstrate. Dopamine- $\beta$ -hydroxylase is found in granules which store epinephrine and has been used as a marker for the vesicular membranes of sympathetic nerves (56).

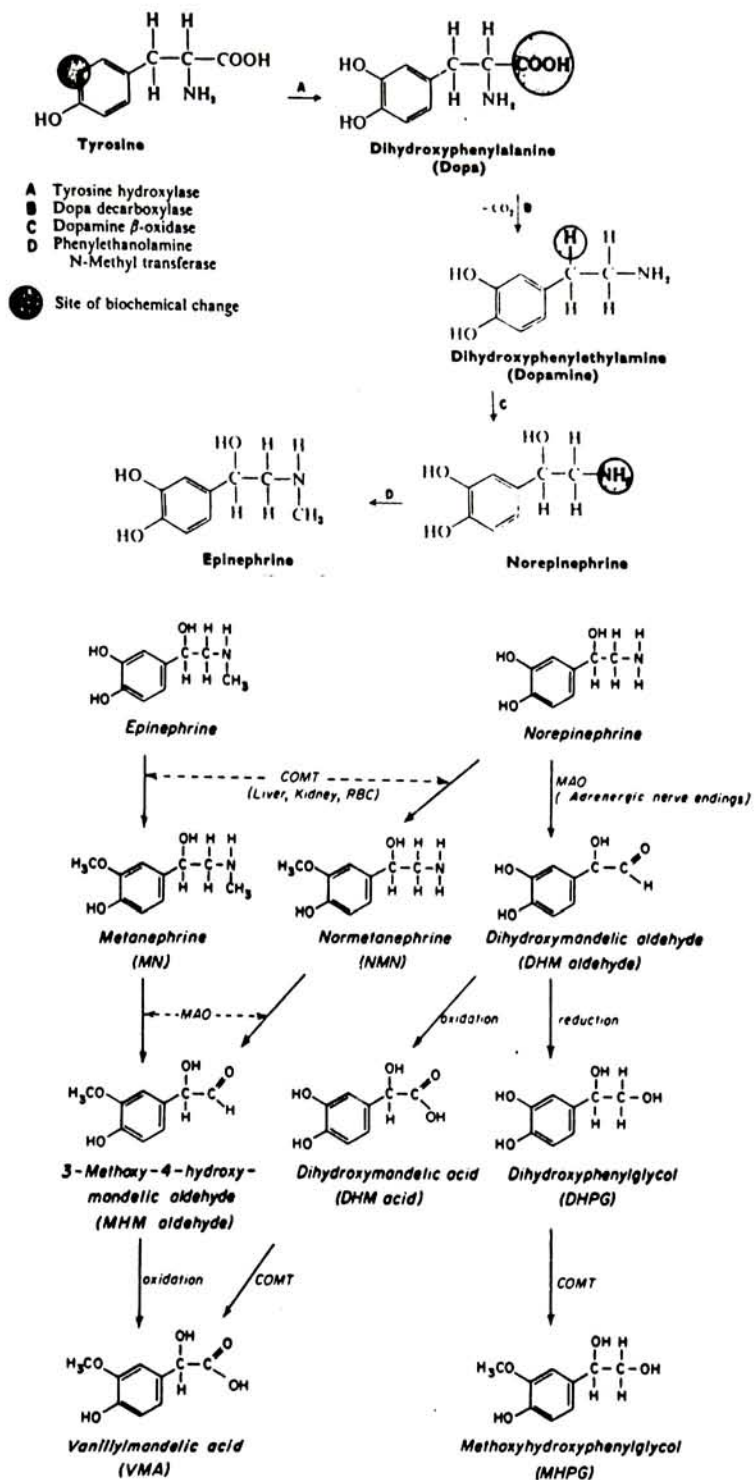


FIGURE 3. Synthetic and degradation pathways of catecholamines (18).



The state of the art alternative for determination of catecholamine is high performance liquid chromatography (HPLC) with electrochemical detection (57). See appendix--Coulochem Application Note.

### **Method of Catecholamine Analysis**

Catecholamine analysis was performed with the Cat-A-Kit<sup>R</sup> (Amersham). This radioenzymatic assay makes use of the enzyme catechol-o-methyl-transferase to catalyze the transfer of <sup>3</sup>H-methyl group from S-adenosyl-L-methionine-(<sup>3</sup>H-methyl)(<sup>3</sup>H-SAM) to norepinephrine, epinephrine, and dopamine. The resulting products are extracted by thin layer chromatography. Isolated derivatives, metanephrine, and normetanephrine are converted by periodate oxidation to <sup>3</sup>H-vanillin. The dopamine derivative, 3-methoxytyramine, is not susceptible to periodate oxidation. This chemical step serves to differentiate dopamine from the other two catecholamines. The commercial protocol appears in the Appendix.

### **Preliminary Investigations**

Preliminary studies were performed to establish quantitative techniques for measuring cell number, viability, catecholamine values, and for documenting changes in cellular morphology.

To control the effect of environmental conditions of the cell culture, pilot experiments were performed to determine the effect of room lights on cells with varying concentrations of the photosensitizer DHE.

Levels of DHE were then chosen to give varying amounts of cytotoxic effect so that cell growth and morphology could be studied.

Finally, since DHE is lipophillic and hydrophobic and sticks together in aqueous solution, it was important to determine that DHE concentration was correct upon dilution. To verify the concentration of DHE, the absorbance of three diluted samples was measured. The concentration was calculated from Beer's law.

### **Catecholamines**

Catecholamine levels were determined in intact cells by fluorescent staining (Appendix), in homogenized cells, and in the medium supernatant. Catecholamine staining was positive in intact C1300 MNB (4B41A3) cells. In cells, quantitative values of catecholamines (Cat-A-Kit<sup>R</sup>) showed the following levels:

**TABLE 2**

## Catecholamine Levels

	<u>Medium†</u>	<u>Homogenized Cells††</u>
Dopamine (DA)(pg/ml)	ND <sup>*</sup>	ND <sup>*</sup>
Norepinephrine (NE)(pg/ml)	41-303	241
Epinephrine (E)(pg/ml)	0-33	303

\* not detectable

† n = 5, 0-6 days

††n = 1

Mouse plasma was assayed for catecholamines following implantation with C1300 NMB (NB412A) cells. Levels were:

**TABLE 3**

## Catecholamine Levels

<u>Control</u>	<u>DA (pg/ml)</u>	<u>E (pg/ml)</u>	<u>NE (pg/ml)</u>
No tumor	8.3	597	2414
n=1			
Tumor	79	26	45
n=1			

The catecholamine levels show that this tumor line produces dopamine in vivo. All catecholamine levels were determined using the Amersham Cat-A-Kit<sup>R</sup> assay at a reference laboratory.

This initial evidence led me to believe there would be enough catecholamine present in medium to measure variation of catecholamine levels following treatment with laser light and DHE.

#### **Characterization of Growth and DHE Toxicity on C1300 MNB Cells - Clone NB41A3**

Normal growth curves were defined for C1300 MNB cells in 48 well plates with no change of medium for 10 days. Cell morphology was observed every day for 10 days. Cells become more spindle shaped and display neurite-like structures on day 3 to day 7. Thus, the earliest day chosen to treat cells with drug and light was day 3, since neurite-like structures are related to the presence of catecholamines.

To determine the DHE toxicity with and without room lights on, cells were grown in 48 well plates and exposed to photosensitizer for two hours. Cells were not washed,



but medium with DHE contained no serum. Results appear in Figures 4 and 5. Toxicity of drug and light were performed with 10 to 50 mW/cm<sup>2</sup> argon laser light and 0.5 to 25 µg DHE/ml with a two-hour incubation time.

## Results

10 mW/cm<sup>2</sup> argon laser and DHE levels of up to 5 µg/ml do not cause cell kill or gross changes in cell morphology. Light levels of >30 mW/cm<sup>2</sup> with DHE levels >10 µg/ml kill all cells present. Therefore, drug levels of 1 and 5 µg DHE/ml and light levels of 30 mW/cm<sup>2</sup> were used to determine sublethal effects on cells in culture.

There is a slight decrease in cell numbers for the concentrations of 5 and 10 µg DHE/ml from day 4 to day 7, as compared to control cells incubated without DHE. The effect of room lights on cell growth is noticeable.

Because of this, the studies on the effects of laser light and DHE on C1300 MNB cells were performed in the dark (1.4 lumens m<sup>-2</sup>), with room lights off and the hood light off.

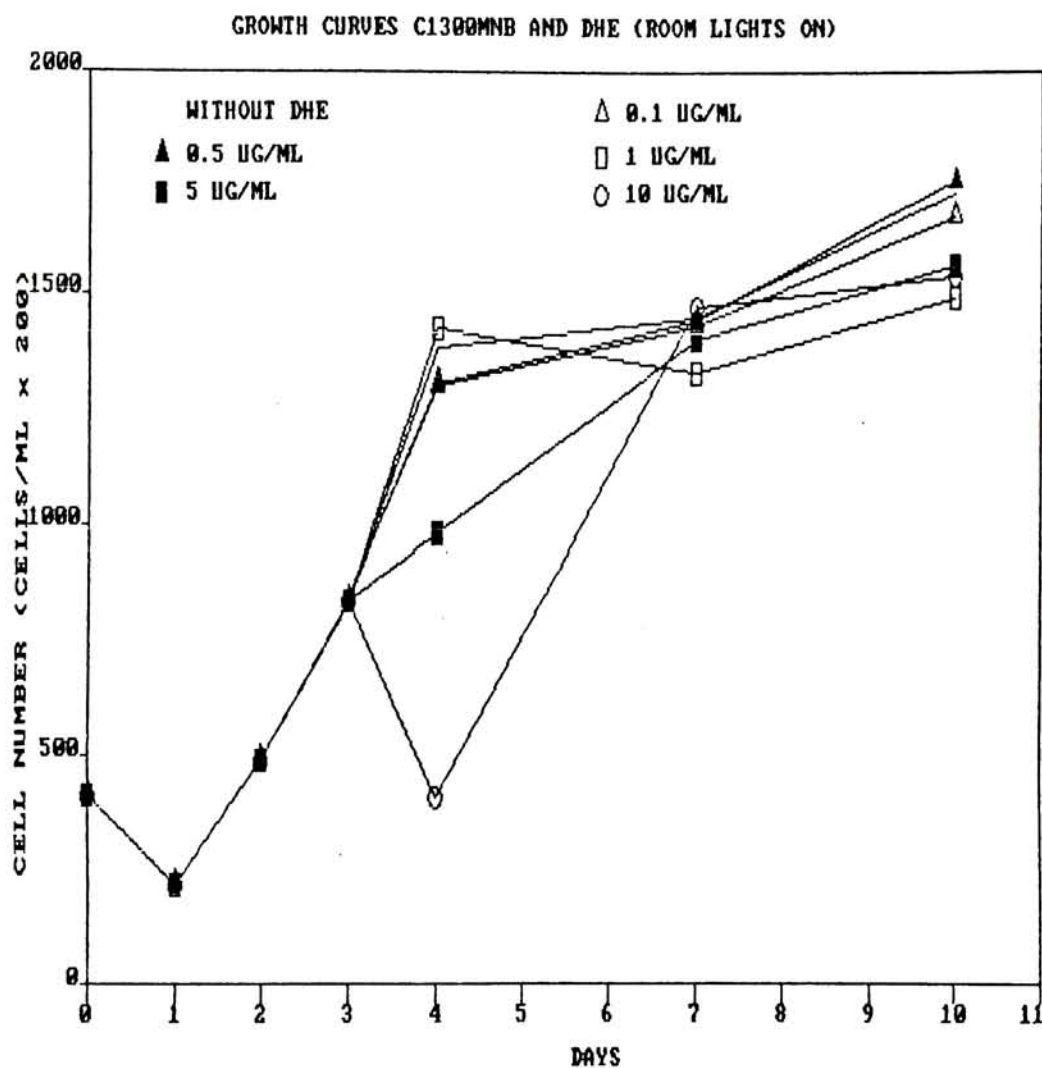


FIGURE 4. Growth curves of C1300 MNB (4B41A3) with different concentrations of DHE and room lights on.

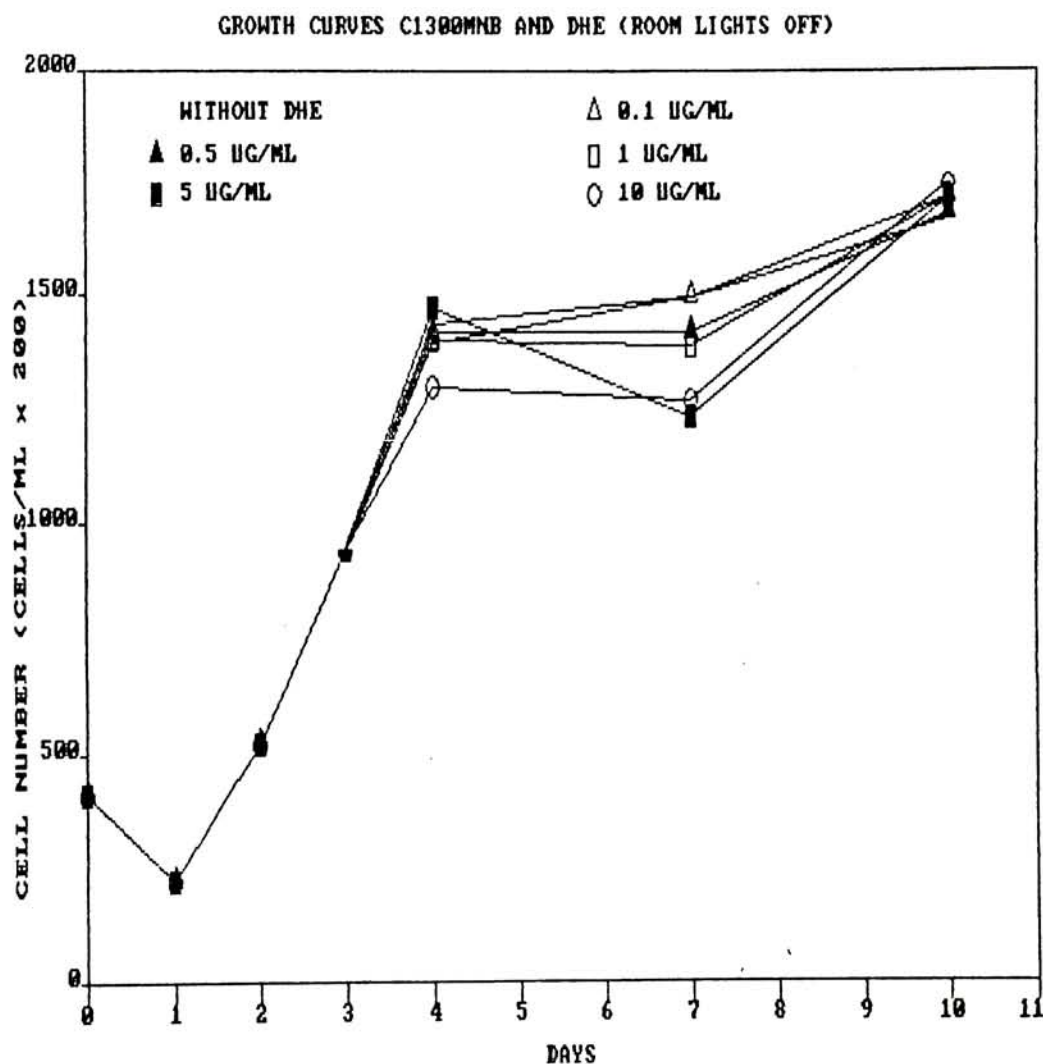


FIGURE 5. Growth curves of C1300 MNB (4B41A3) with different concentrations of DHE in the dark.

## Results of Confirming Accuracy of Dilution of DHE

DHE is lipophilic and hydrophobic and may stick together, leading to inaccurate dilutions. Using Beer's law, the concentration of DHE in solution can be determined by measuring the absorbance.

Beers's Law:

$$A = abc$$

where  $A$  = absorbance,  $a$  = molar absorptivity coefficient (or  $E$ , extinction coefficient),  
 $b$  = 1 cm path length of sample cell, and  
 $c$  = concentration in moles or grams.

Physical and chemical constants for HPD and DHE are:

DHE  $a = 5000 @ 506 \text{ nm}$

HPD  $E = 0.126 \text{ cm}^{-1} \mu\text{g}^{-1} @ 405 \text{ nm}$

DHE molecular weight = 627

Reorganizing Beer's law equation for concentration:

$$c = A/ab$$

Using this equation and physical chemistry data given, the concentration of each DHE dilution can be calculated from the absorbance value obtained from a spectrophotometer.

## Results

The results are tabulated below and shown graphically in Figure 6. These results show that DHE is soluble in medium. When diluted, the measured concentrations match the calculated values. Measuring the absorbance at the 405 nm wavelength gives values closer to the calculated values than when measured at the 506 nm wavelength. If the linear results at 405 nm were correct, DHE absorbs light like HPD. If the linear results at 506 nm were correct, then DHE does aggregate. I assumed absorptivity at 405 nm was correct because DHE has 20% HPD.

## The Experiment - An Overview

The purpose of the experiment is to determine the effect of argon laser and the photosensitizer DHE and a combination of both, on C1300 MNB cells. C1300 MNB cells isolated from fresh tumor removed from mice were grown in tissue culture flasks for three to four days and passed in tissue culture. Cells were plated at a concentration  $6 \times 10^4$  cells per milliliter into 48 well tissue culture trays. Cells were grown 72 hours in Ham's F10 medium with glutamine, 2.5% Fetal Bovine Serum, and 15% Horse serum. At 72 hours, cells were washed once with

TABLE 4

DHE Calculated Concentration $\mu\text{g/ml}$	Measured			Conc. DHE mols/L	$A_{506}$	$\mu\text{g/ml}$	Conc. DHE mols/L	$A_{405}$
	$\mu\text{g/ml}$	Conc. DHE mols/L	$\mu\text{g/ml}$					
1.0	2.38	$1.595 \times 10^{-6}$	0.019	$3.8 \times 10^{-6}$	0.019	0.928	$3.8 \times 10^{-6}$	0.117
5.0	8.90	$7.975 \times 10^{-6}$	0.071	$1.42 \times 10^{-5}$	0.071	4.603	$1.42 \times 10^{-5}$	0.580
10.0	19.31	$1.595 \times 10^{-5}$	0.154	$3.08 \times 10^{-5}$	0.154	10.1260	$3.08 \times 10^{-5}$	1.276



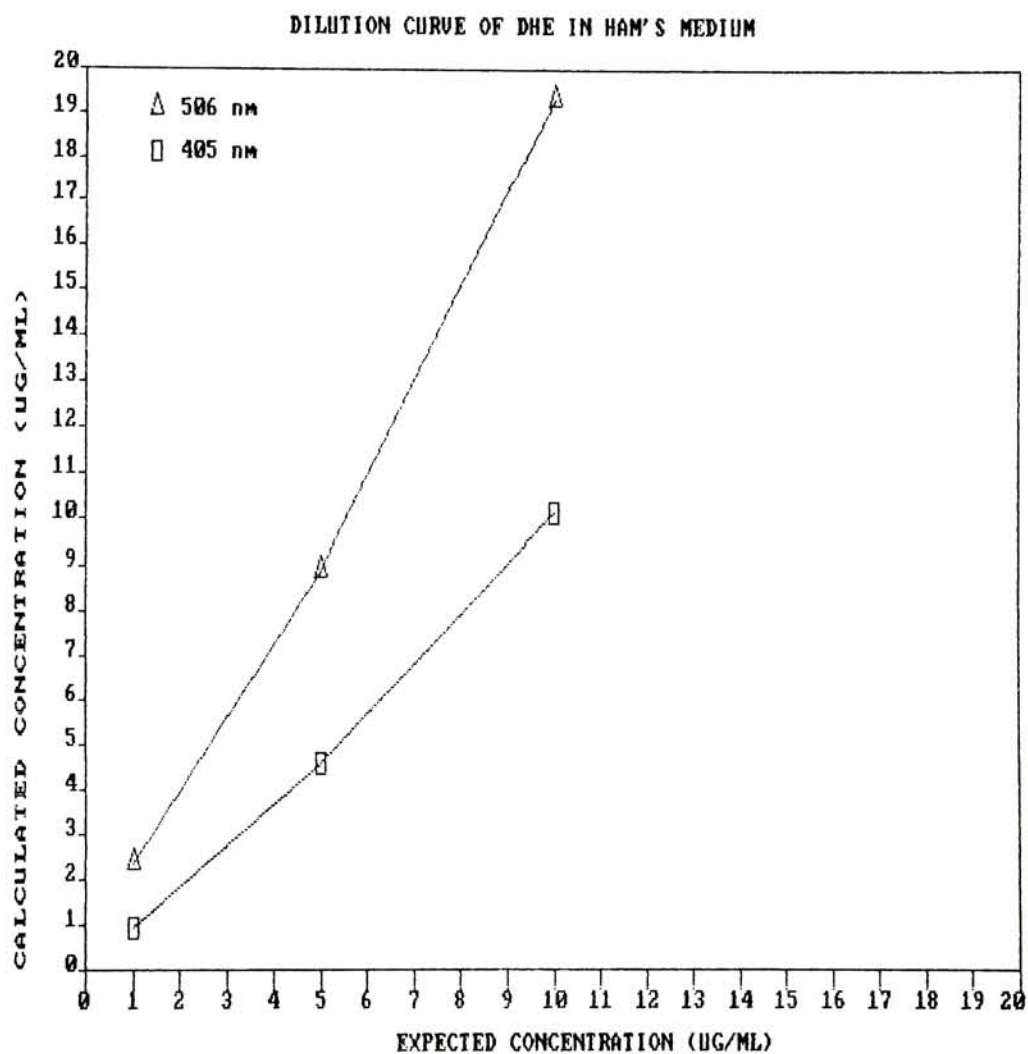


FIGURE 6. Dilution curve of DHE in Ham's F10 medium.

magnesium and calcium free Phosphate Buffered Saline (PBS) to remove serum. Ham's F10, without serum, with or without DHE, was added to the appropriate treatment or control well for a two hour, 37°C incubation. Following the two hour incubation, cells were washed once with calcium and magnesium free PBS to remove residual DHE.

Fresh growth medium with supplements was added to all wells. The plates were treated with 30 mW cm<sup>-2</sup> argon laser light or left in the dark. One hour post irradiation, cells were sampled for cell number, viability, and supernatant medium saved for catecholamine analysis. Wells in each group were sampled on days 3, pre and post treatment, 4, 7, and 9. Photographs were taken at each sampling interval to document cell morphology.

## MATERIALS AND METHODS

### Cell Line

Mouse neuroblastoma C1300 MNB - NB41A3 (ATCC-CCL-147) was obtained from American Tumor Cell Collection. Tumor was implanted subdermally in A/J male (Jackson Laboratories, Bar Harbor, ME) mice six to eight weeks old. After 14 to 21 days, following sacrifice of the animals by cervical dislocation, tumors were excised. Prior to excision of the tumor, dead rats were dipped into alcohol and then skin over the tumor was shaved. The tumor was removed and trimmed of necrotic and non-tumor tissue. The tumor was strained through a sterile stainless steel mesh screen into a sterile petri dish containing Ham's F10 medium (Flow Laboratories, McLean, VA) with 2.5% Fetal Bovine Serum, 15% Horse Serum, 6  $\mu$ M glutamine, and 100 units per milliliter of penicillin and streptomycin (Flow Laboratories, McLean, VA). Cells were plated into T-25 Falcon culture flasks (Fisher Scientific, Rochester, NY) and passaged routinely at 1:3 ratio twice a week to ensure viability and exponential growth. Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

## DHE - The Photosensitizer

Dihematoporphyrin ether (DHE, Photofrin 2<sup>R</sup>) was obtained from Photofrin Medical, Inc. (Raritan, NJ). Four vials of DHE (Lot 249C) were stored at -80°C. Each vial contained 30 ml of DHE at a concentration of 2.5 mg/ml. Prior to use, a vial was thawed and 4 ml portions were pipeted into 17 x 100 mm sterile polypropylene test tubes with caps (Fisherbrand, Fisher Scientific, Rochester, NY). Aliquots were stored at -80°C until use. Lot 249C of DHE was used for the entire experiment.

Serial dilutions of 1.0 and 5.0 µg DHE per milliliter in Ham's F10 medium without serum were made from freshly thawed DHE stock (2.5 mg/ml). Dilution-concentration curves were determined by measuring absorbance and calculating actual concentration from Beer's law. Preliminary studies have shown DHE has an extinction coefficient the same as hematoporphyrin derivative (photofrin 1), which is  $0.126 \text{ cm}^{-1} \mu\text{g}^{-1}$ .

DHE stock and working solutions were kept in the dark. One milliliter of Ham's F10 medium containing DHE was added to the appropriate treatment well two hours prior to irradiation with laser light.

## **Argon Laser and DHE Effects on Morphology, Growth, and Viability**

Cells growing exponentially in three to four day old culture flasks were displaced by multiple washing with fresh medium. Cell numbers (cells/ml) in the stock culture were determined by counting on a Coulter Counter Model ZF (Coulter Electronics, Inc., Hialeah, FL). The stock solution of cells was diluted with medium to  $6 \times 10^4$  cells per milliliter. Actual cell number was determined by automatic counting before plating. One milliliter of  $6 \times 10^4$  cells was added to each of 18 wells of a 48 well tissue culture cluster plate (Costar, Cambridge, MA). Two 48 well plates were set up per replicate. Cells were grown for three days prior to treatment. At three to seven days, the cells were round and spindle shaped with neurites.

Observation of cell morphology, cell counts, and viability assessment were performed on replicates on days 2 and 3 pre-treatment, and days 3, 4, 7, and 9 post-treatment.



## **Cell Morphology**

Morphology of cells in each well at each time interval was assessed with the aid of a Nikon inverted phase microscope at 200x magnification. Photographic records were made with an Olympus OM-1 35 mm camera attached to the microscope via a Nikon extender tube. Kodak 200 ASA color slide film was used. A photograph was taken of cells in each well. Then cell counts were made of the attached cells. Cell medium was removed and saved for catecholamine analysis or for viability studies.

## **Cell Counting**

Cells were detached from the surface of each well of the 48 well plate by incubating them for 10 minutes in 0.2 milliliters of trypsin-EDTA (Flow Laboratories, McLean, VA) at room temperature. After 10 minutes, 0.3 milliliters of calcium and magnesium free phosphate buffered saline (PBS) was added to each well to stop trypsinization and to bring the volume to 0.5 milliliter.

Cells and trypsin-PBS solution were removed from wells by scraping the well bottom with a disposable pipet tip that was attached to a 1000  $\mu$ l Socorrex variable



pipet (VWR, San Francisco, CA). This pipet was used to transfer well contents to culture tubes by first refluxing medium five times to free and mix cells. Cells were removed from wells and placed into 12 x 75 mm round bottom non-sterile polypropylene culture tubes (VWR, San Francisco, CA).

Cell counts were performed on the Coulter counter following a 1:100 dilution ( $200\ \mu\text{l}/20\ \text{ml}$ ) using a Coulter diluter II (Coulter Electronics, Inc., Hialeah, FL). Diluent with cells was dispensed into 30 ml sample vials. Sample vial was placed on the counter and 0.5 ml was aspirated. The readout displayed the absolute count of cell numbers. This number was multiplied by 200 to get cell number per milliliter.

### **Cell Viability**

Cell viability was determined by trypan blue exclusion. Following sampling for cell counting, the remaining cell solution in the 12 x 75 mm culture tube was used for viability studies. One drop of filtered 0.16% Trypan Blue was added to the culture tube with a disposable pasteur pipet.

The sample was mixed briefly on a maxi mix 1 multitube vortex mixer (Thermolyne, Dubuque, IA). One drop of each sample was placed onto a glass microscope slide inside a 1 cm diameter ink circle. The ink circle retains the drop. Cells were allowed to settle for three to five minutes. One photomicrograph each was taken of two fields of cells in each ink circle. The microscope camera set-up was used, as described for photodocumentation of cell morphology. Live and dead (blue) cells were counted, from the projected color slides to determine cell viability of each well. Cell viability was reported as percentage--live to total cell number.

## **Treatment**

The cells were treated on the third day after having been plated into 18 wells, 4 wells at each corner and 2 central wells, of two 48 well plates. Immediately prior to treatment, medium was removed from all wells with a sterile pasteur pipet attached via tubing to a suction flask. Cells in all wells were washed with one milliliter of 37°C PBS with calcium and magnesium to remove serum. PBS was removed with a pasteur pipet and suction.

One milliliter of Ham's F10 medium with glutamine without serum was added to both the control and the "light only" treatment wells. The room lights and hood lights were turned off. Light levels were measured with a Gossen luna pro light meter (West Germany). Light level was 350 lumens  $m^{-2}$  with the lights on and  $\leq 1.4$  lumens  $m^{-2}$  with the room lights off. One milliliter of Ham's F10 with glutamine without serum and with either 1.0 or 5.0  $\mu g$  DHE per milliliter was added to wells of "DHE only control" and "photodynamic therapy - light and DHE" groups.

Plates were incubated for two hours in total dark at 37°C in a humidified 95% air and 5% CO<sub>2</sub> environment. Immediately following the two-hour incubation, photomicrographs were taken of one well in each group for documentation of cell morphology.

All wells were washed with 1 ml, 37°C PBS with calcium and magnesium. Then one milliliter of Ham's F10 medium with serum was added to each well.

Plates with control and DHE only wells were placed in the tissue culture hood in the dark at room temperature. Plates with light only control wells and

PDT wells were irradiated via a 600  $\mu\text{m}$  glass fiber (Cooper Lasersonics, Santa Clara, CA) with 30  $\text{mW}/\text{cm}^2$  argon laser (Lexel Model 550, Cooper Lasersonics, Santa Clara, CA) light for one minute ( $1.8 \text{ Joules}/\text{cm}^2$ ) and five minutes ( $9.0 \text{ Joules}/\text{cm}^2$ ) in a darkened room at room temperature. Immediately following treatment, photomicrographs were taken of one well in each group to document cell morphology. All plates were returned to the  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator for one hour.

After one hour, the medium was removed from one well in each group and it was saved for catecholamine analysis. The trypsin was added to one well in each group to release cells from the plate surface, followed by cell counts and trypan blue viability determinations. The plates were returned to the  $37^\circ\text{C}$  incubator for six days. All photomicrographs, cell counts, trypan blue viability determinations, and cell washings were performed in the darkened room.

In pilot studies, temperature of the medium was measured during the time of irradiation with the argon laser. No temperature change occurred.



Experiments were run in duplicate for "control" and "DHE only" groups and one repetition for each light only group - 1 and 5 minutes, and each light and DHE combination (PDT). Fourteen replicates of each experiment were performed.

### **Laser Dosimetry and Beam Profiling**

The argon ion laser outputs light at 488 nm and 514 nm. Power output of the argon laser from the fiber tip was measured with the Liconix PM45 power meter (Liconix, Sunnyvale, CA). This power meter has a photoreceptor cell calibrated to each of four wavelengths. The power meter was set at a range of 30 mW, 514 nm wavelength. The photoreceptor cell has an aperture size of  $0.38 \text{ cm}^2$ . Therefore, to determine power in milliwatts per  $\text{cm}^2$ , the meter reading is multiplied by 2.63. The irradiation took place in an air regulated incubator. The fiber was placed 25 centimeters below the plate holder so that a spot or beam diameter of 7.0 cm was obtained. Beam sighting was enhanced by reflecting the beam off a card coated with fluorescein. The diameter was measured across the homogeneous beam cross section.

Power measurements were made at the center point and at each quadrant, 1 cm from center, and 3.5 to 4 cm from center. In the 1.5 cm diameter circle that covered the four simultaneously treated wells, the power level was maintained between  $30.25 \text{ mW/cm}^2$  and  $32.8 \text{ mW/cm}^2$  for all irradiations. Four corner wells were irradiated at once within the 1.5 cm diameter circle. For one minute of argon exposure, the energy density is  $1.8 \text{ Joules cm}^{-2}$  and for five minutes is  $9.0 \text{ Joules cm}^{-2}$ .

### **Catecholamine Analysis**

Samples of growth medium were obtained from wells containing C1300 MNB cells at each time interval and from all groups prior to cell counting. One milliliter of medium was removed from each well using a sterile five-milliliter serological pipet (Fisher Brand, Fisher Scientific, Rochester, NY) and transferred to an ice cold four-milliliter blood collection tube (Amersham, Arlington Heights, IL) containing EGTA (ethylene glycol-bis (B-aminoethyl ether)  $N,N^1$ -tetra acetic acid) and reduced glutathione. Immediately thereafter, the tubes were centrifuged for 10 minutes at 800 g. Then cell-free medium was decanted into 17 x 75 millimeter clear polystyrene test tubes (Falcon 2054, Fisher Scientific, Rochester, NY) capped and immediately frozen to  $-80^{\circ}\text{C}$ .



The differential catecholamine determination was performed using the radioenzymatic assay system (Cat-A-Kit<sup>R</sup>, Amersham). Medium from one replicated each, using 1 µg DHE/ml and 5 µg DHE/ml as treatment concentrations, was sampled for dopamine, epinephrine, and norepinephrine. Samples were thawed immediately prior to analysis and kept on ice. Samples were run in quadruplicate with two tubes for sample alone and two tubes with samples spiked with 100 pg/ml of dopamine, epinephrine, and norepinephrine. The complete step by step protocol and materials list appears in the appendix.

### **Application Notes**

The samples were reacted with the enzyme isotope mixture for one hour in a shaking water bath. After this reaction and all extractions, sample catecholamines in acetic acid were frozen to -80°C until the next day. Thin layer chromatography was performed. Separation took 60-90 minutes for four plates in a glass developing tank. The tank had saturation pads and a solvent volume of 88 milliliters.

Prior to counting on a Beta counter, all vials were shaken for 45 seconds and dark adapted at least two hours.

Vials were counted on a refrigerated Packard 460C (Downers Grove, IL) liquid scintillation counter.

Results were reported in picograms of catecholamine per milliliter (pg/ml). Concentration of each catecholamine was determined by the following formula:

$$\text{Concentration (pg/ml)} = \frac{\text{CPM}^* \text{ sample} - \text{CPM Blank}}{\text{CPM standard} - \text{CPM Sample}} \times \frac{\text{Standard } 100 \text{ pg/ml}}{0.050 \text{ ml}}$$

\* CPM = counts per minute

## STATISTICAL ANALYSIS

Distribution and comparative statistics -- ANOVA, the Student's t-test and graphical display of variance were used to define and compare cell count and viability data within and between treatment groups.

All statistical computations were performed using Statpro<sup>R</sup> (Penton Software, NY, NY) software.

### Quality Control

Cell counting precision and accuracy determined by measuring the level of 14.7  $\mu\text{m}$  microspheres (Coulter Electronics, Hialeah, FL) in solution. Microsphere counts were determined every Monday. The mean coefficient of variation of triplicate cell counts was reported.

Catecholamine precision and accuracy was determined by measuring the level of dopamine, epinephrine, and nor-epinephrine in human plasma control provided in the assay kit.

## RESULTS

### Cell Morphology

The appearance of cells in the tissue culture well at various times pre and post treatment gives a qualitative picture of cell status. Photomicrographs from an experimental replicate, using DHE 5  $\mu$ g/ml, show characteristics of shape, size, density, and cellular inclusions. These photomicrographs are representative of the cell morphology of all experimental replicates. These are photomicrographs of the "control groups" pre and post treatment and of the "worst case" treatment groups.

Cells were harvested from stock cultures and diluted to  $6 \times 10^4$  cells per milliliter. One milliliter was added to a well that has a surface area of 1 square centimeter.

Characteristic morphology of untreated cells, 48 and 72 hours after plating, appear in Figures 7A and 7B. At day 2 (Figure 7A), cells attached to the plate have started to divide and elongate--spindle cells. Neurite-





FIGURE 7A. Cell morphology Day 2  
pre treatment.

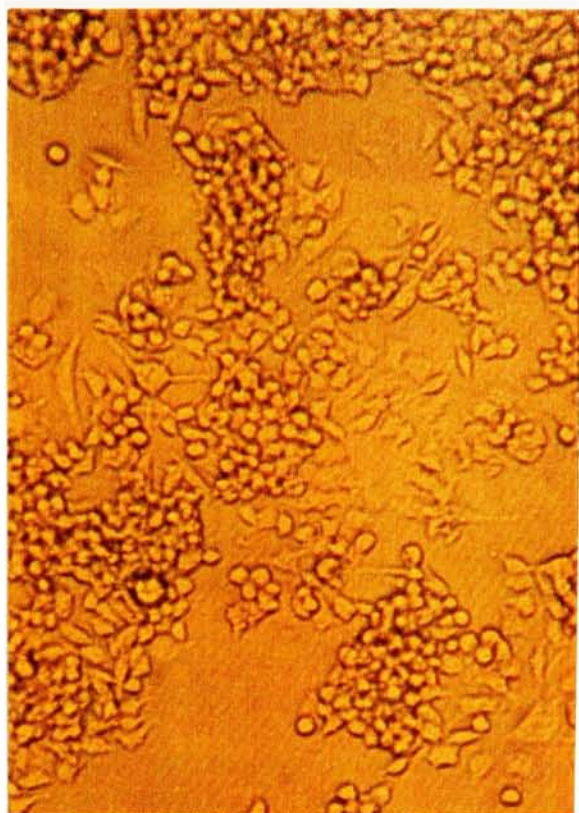


FIGURE 7B. Cell morphology Day 3  
pre treatment.

like structures began to appear on spindle cells. At day 3 (Figure 7B), more cells are evident and cells are assuming the characteristic shape of nerve cells, with long dendritic structures. The cytoplasm is clear.

Cell morphology changes with time and after treatment. All wells, thus all cells, were washed once prior to treatment with photosensitizer to remove serum proteins from cells. The cells were washed a second time following the two-hour incubation with DHE and without DHE. This wash was to remove residual DHE from cells.

Photomicrographs were taken of cell morphology of "control"--nontreated, light only, and DHE only groups--and of treated--light and DHE--at times immediately (Day 3) after, and at 1 hour (Day 3), 24 hours (Day 4), 96 hours (Day 7), and 144 hours (Day 9) post irradiation with the argon laser. All photomicrographs were taken at 200x magnification unless noted otherwise.

Figures 8A-F show the changes in cell morphology for the control, DHE only-5  $\mu$ g/ml, light only-1 minute, light only-5 minutes, light-1 minute and DHE-5  $\mu$ g/ml, and light-5 minutes and DHE-5  $\mu$ g/ml.



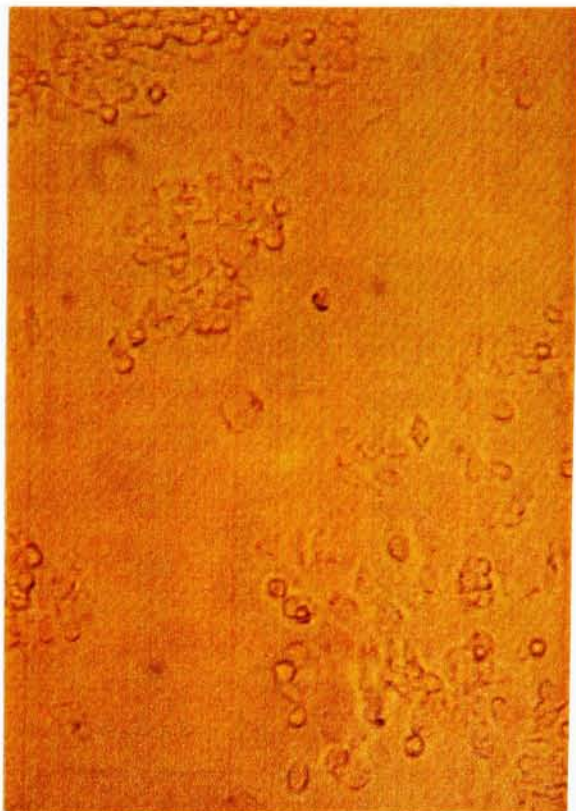


FIGURE 8A. Cell morphology of **control** immediately post treatment. Day 3.

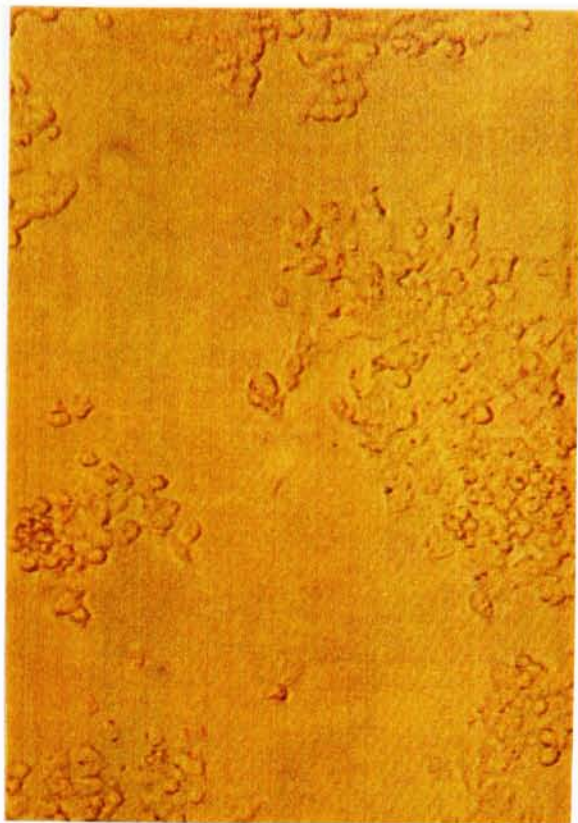


FIGURE 8B. Cell morphology of **DHE** only **5  $\mu\text{g/ml}$**  immediately post treatment. Day 3.

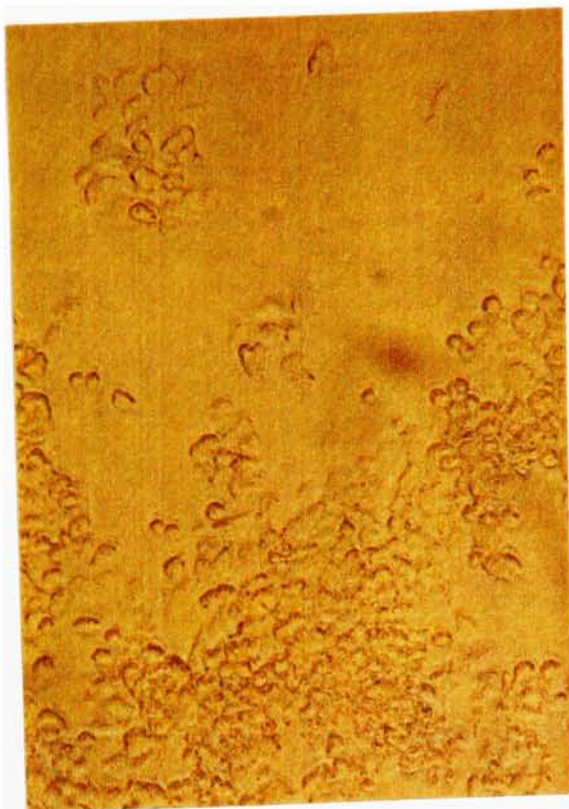


FIGURE 8C. Cell morphology of Light only 1 minute immediately post treatment. Day 3.



FIGURE 8D. Cell morphology of Light only 5 minutes immediately post treatment. Day 3.





FIGURE 8E. Cell morphology of Light 1 minute and DHE 5  $\mu$ g/ml immediately post treatment. Day 3.



FIGURE 8F. Cell morphology of Light 5 minutes and DHE 5  $\mu$ g/ml immediately post treatment. Day 3.

In the "control" groups, A-D, cell washing has reduced the number of cells. The cells were less spindle shaped because of being less attached. Dark granules were seen in a few cells in Figure 8B, DHE only control. Light only at both 1 and 5 minutes increased the number of vacuoles in the cells. Overall, all cells in these groups appear normal.

The morphology of cells in the light and DHE-5  $\mu\text{g}/\text{ml}$  treatment groups immediately following irradiation detached from the cell surface and become rounded. Cells appear pyknotic--granular with dense and frequent inclusions. Laser light and DHE treated cells were injured or killed, causing the observed effects on cell morphology.

There was no difference of cell morphology between groups treated with 1 minute and 5 minutes of laser light at the DHE 5  $\mu\text{g}/\text{ml}$  level and light-5 minutes and DHE 1  $\mu\text{g}/\text{ml}$  level. Morphology of cells treated with 1 minute argon laser light and DHE 1  $\mu\text{g}/\text{ml}$  was no different than light-1 minute alone.

After treatment, cells were incubated at 37°C in a 5% CO<sub>2</sub> environment. At one hour post treatment, cell

morphology appears the same as immediately after treatment, except in the light only-5 minutes and treated groups. In the light only-5 minutes group, cells are rounded and small--appearing to be injured or dead. In the "light-1 minute and DHE 5  $\mu$ g/ml" group, cells are smaller, appearing dead. Figure 9F shows cell morphology at 300x magnification.

In the "control" groups (Figures 10A-D), at 24 hours post treatment, cells had increased in number and started to show characteristic spindle shape. Some cells started to form neurite-like structures. Cell morphology in the light only-5 minutes group showed dense cell numbers the same as in the untreated, DHE only, and light only-1 minute groups. In the light and DHE treated groups (Figures 10E-F), cells that were injured were still rounded and dead cells were disintegrating. A few cells started to recover but still were pyknotic.

Cell morphology appears in Figure 11A-C at 96 hours (day 7) and Figures 12A-C at 144 hours (day 9) post treatment. With increasing time, cells in the "control" group became more differentiated--neuron-like. Figures 11A and 12A show representative examples of cell morphology.



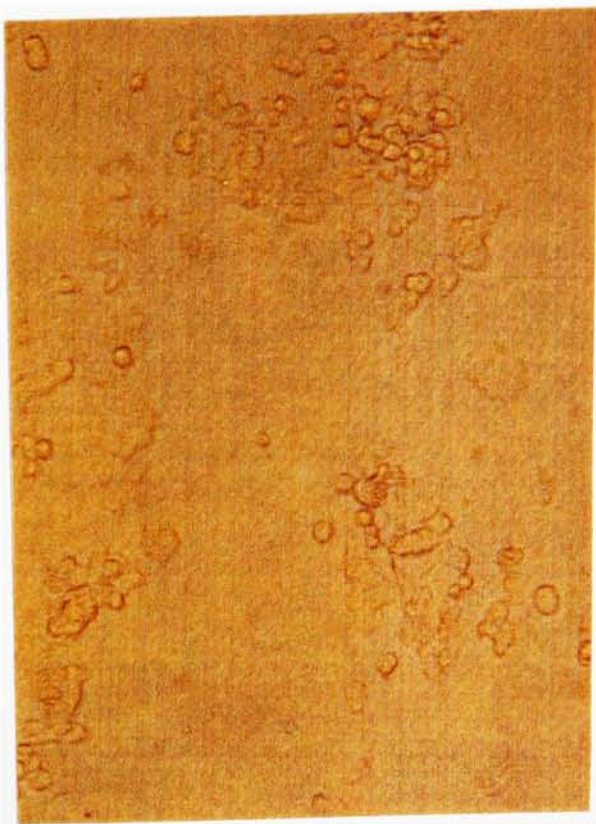


FIGURE 9A. Cell morphology of **control**  
1 hour post treatment.  
Day 3.

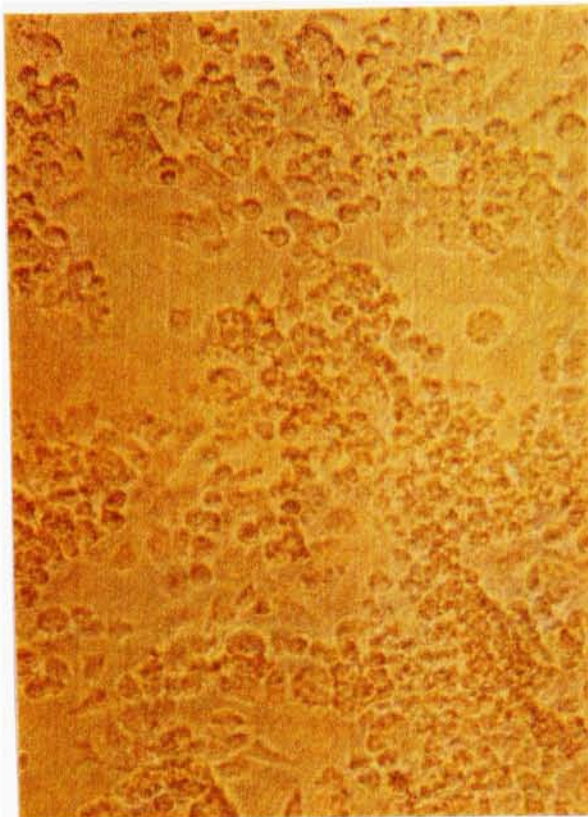


FIGURE 9B. Cell morphology of **DHE**  
**only 5  $\mu\text{g/ml}$**  1 hour post  
treatment. Day 3.





FIGURE 9C. Cell morphology of Light  
only 1 minute 1 hour post  
treatment. Day 3.

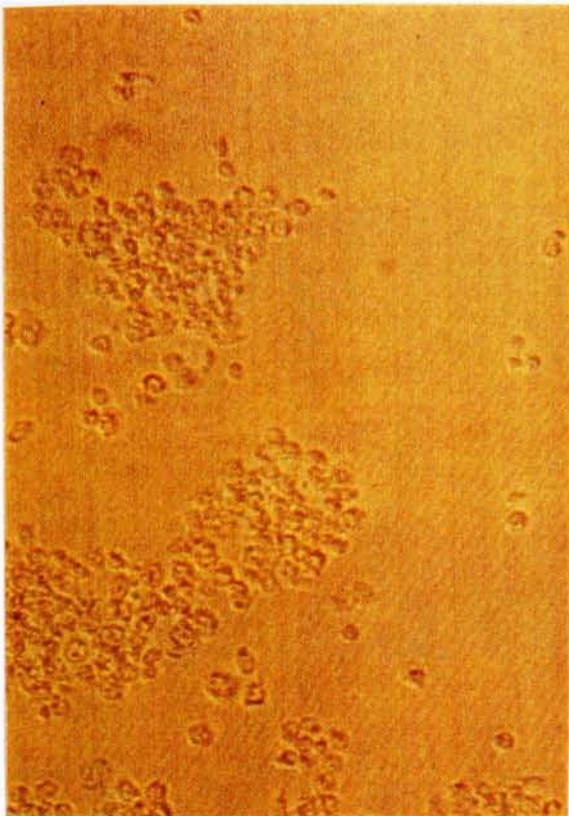


FIGURE 9D. Cell morphology of Light  
only 5 minutes 1 hour post  
treatment. Day 3.

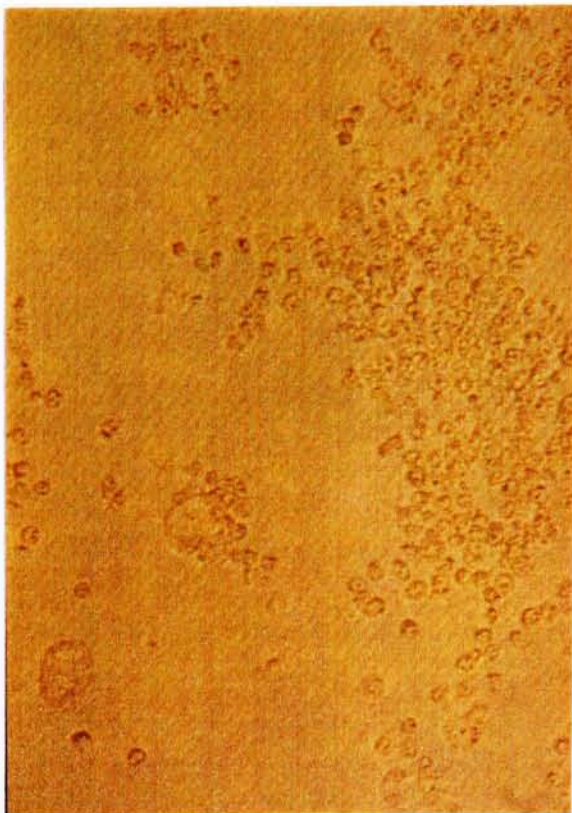


FIGURE 9E. Cell morphology of Light  
1 minute and DHE 5  $\mu\text{g/ml}$   
1 hour post treatment.  
Day 3.

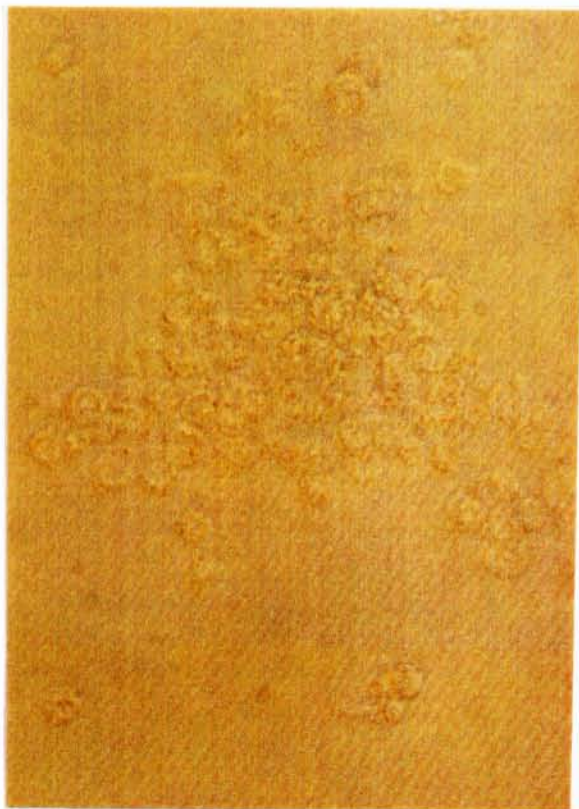


FIGURE 9F. Cell morphology of Light  
5 minutes and DHE 5  $\mu\text{g/ml}$   
1 hour post treatment.  
Day 3. (300x)





FIGURE 10A. Cell morphology of **control**  
24 hours post treatment.  
Day 4.

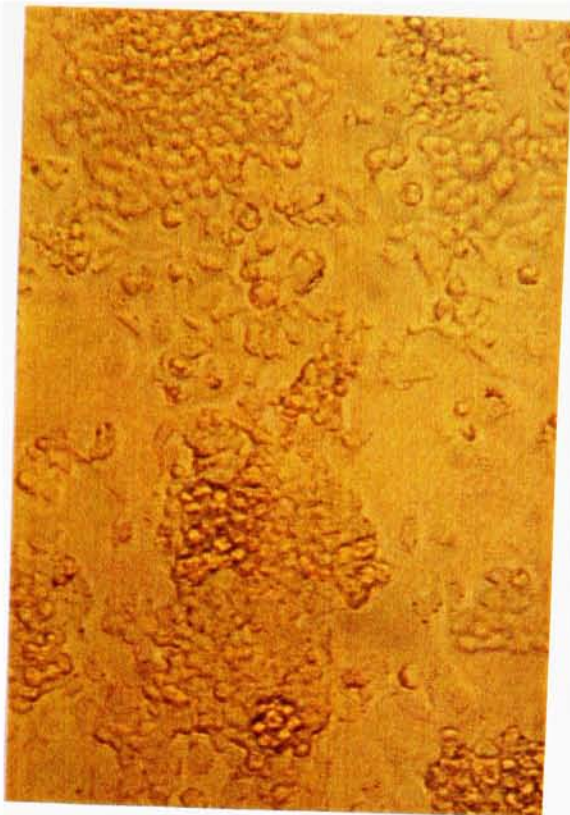


FIGURE 10B. Cell morphology of **DHE**  
**only 5  $\mu\text{g/ml}$**  24 hours post  
treatment. Day 4.



FIGURE 10C. Cell morphology of Light  
only 1 minute 24 hours post  
treatment. Day 4.



FIGURE 10D. Cell morphology of Light  
only 5 minutes 24 hours post  
treatment. Day 4.



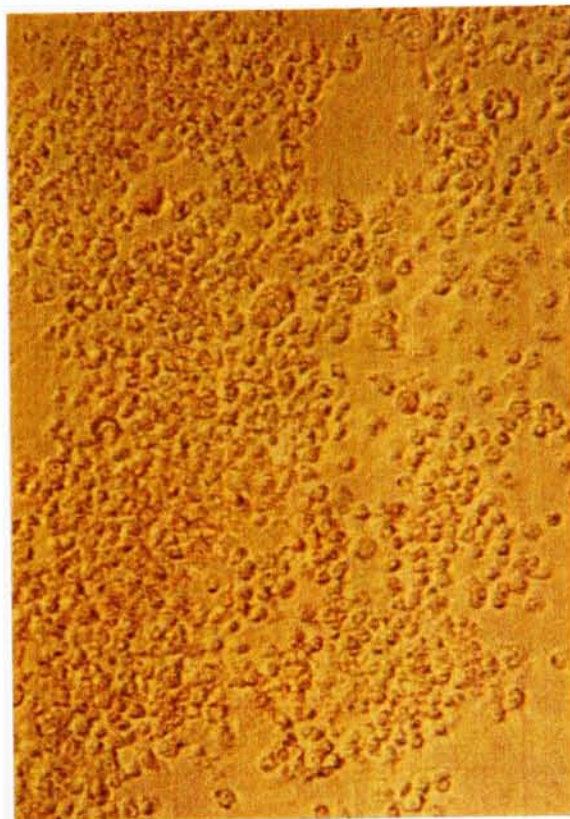


FIGURE 10E. Cell morphology of Light  
1 minute and DHE 5  $\mu\text{g/ml}$   
24 hours post treatment.  
Day 4.

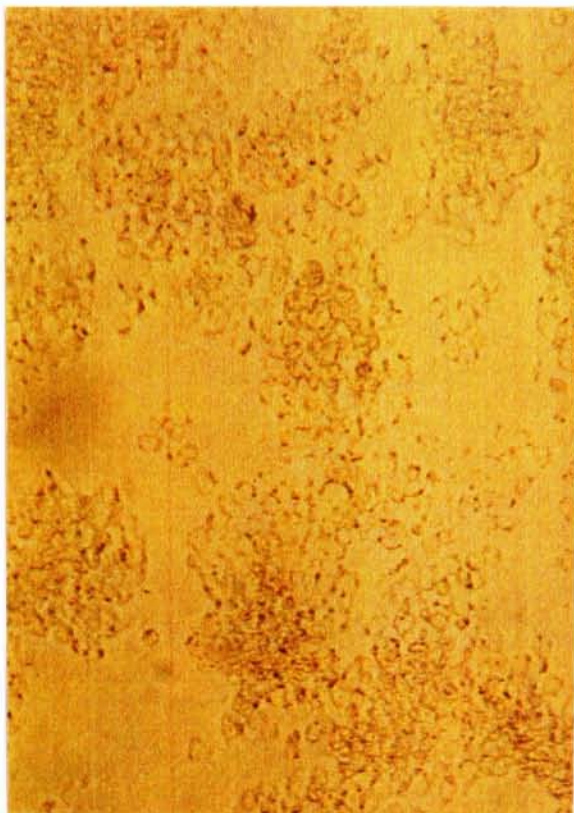


FIGURE 10F. Cell morphology of Light  
5 minutes and DHE 5  $\mu\text{g/ml}$   
24 hour post treatment.  
Day 4.

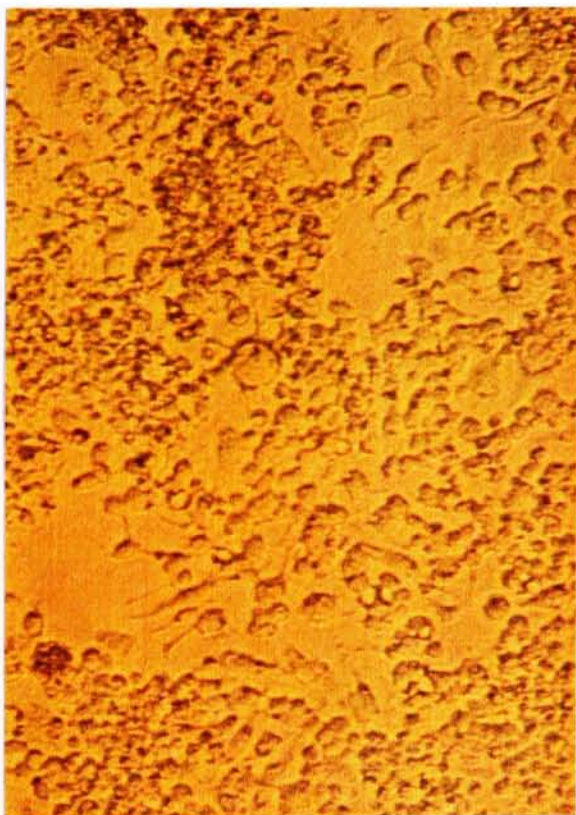


FIGURE 11A. Cell morphology of **control**  
96 hours post treatment.  
Day 7.

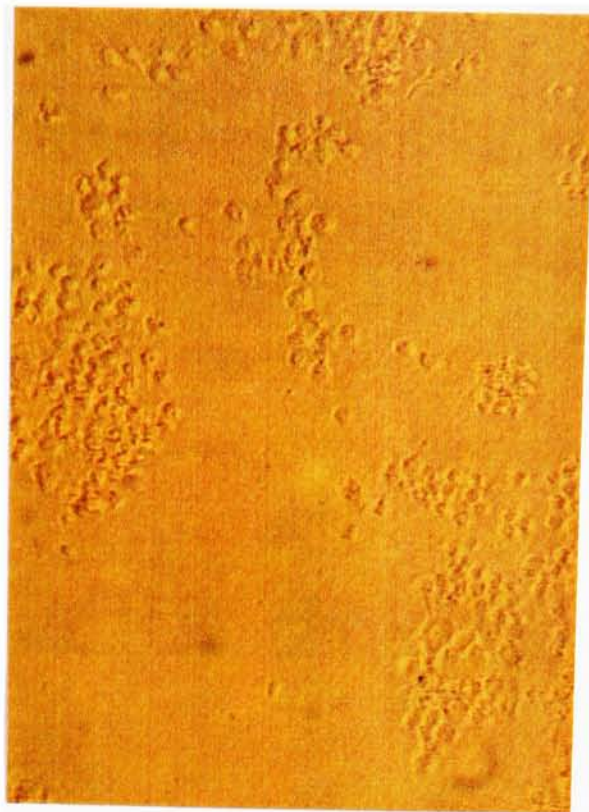


FIGURE 11B. Cell morphology of **Light 1**  
**minute and DHE 5 µg/ml** 96  
hours post treatment. Day 7.



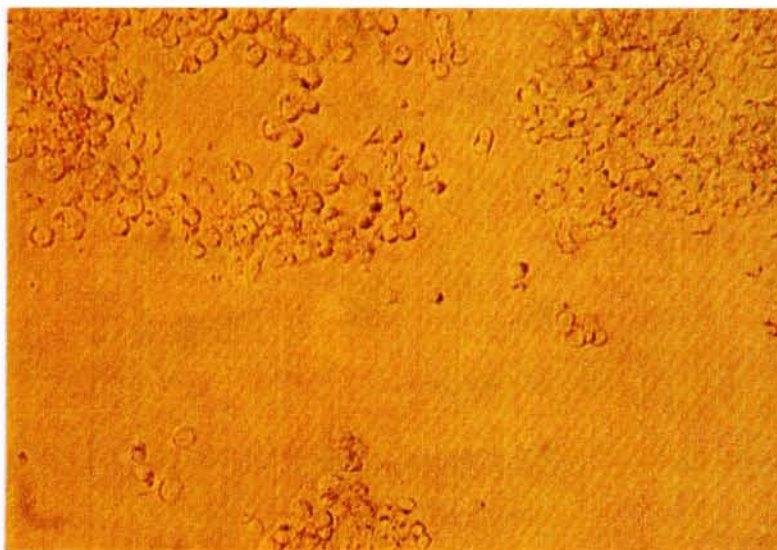


FIGURE 11C. Cell morphology **Light 5 minutes and**  
**DHE 5  $\mu$ g/ml** 96 hours post treatment.  
Day 7.



FIGURE 12A. Cell morphology of **control**  
144 hours post treatment.  
Day 9.



FIGURE 12B. Cell morphology of **Light**  
**1 minute and DHE 5 µg/ml**  
144 hours post treatment.  
Day 9.

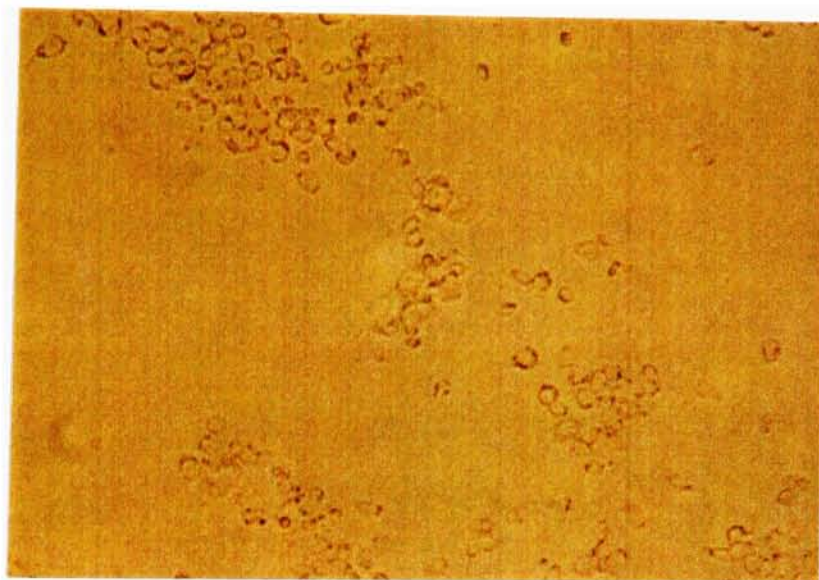


FIGURE 12C. Cell morphology **Light 5 minutes and**  
**DHE 5  $\mu$ g/ml** 144 hours post treatment.  
Day 9.

Cells that survived the treatment were observed in the light and DHE treated groups (Figures 11B-C and 12B-C). Most were rounded with unpaired cell function. A few recovered to show normal cell morphology.

### **Cell Growth**

Living cells reproduce exponentially in tissue culture wells. Cell counting at selected time intervals gave a quantitative measure of growth. Cell counts were performed on cells from all wells of each replicate. Results are compiled in Table 5. These results are represented graphically in Figure 13.

### **Experimental Design**

Each experimental replicate ran over nine days. Each replicate had two untreated control four-well clusters, two DHE only four-well clusters, and one four-well cluster for light-1 minute, light-5 minutes, and one cluster each for the light and DHE treated groups. The four-well cluster represented one well for each day sampled post treatment.



TABLE 5  
CELL COUNTS AS COMPARED OVER TIME

Day	Control	Day	Control	DHE (1 <sub>u</sub> g/ml)	DHE (5 <sub>u</sub> g/ml)	Light 1 min.	Light 5 min.	L 1 min. DHE 1 <sub>u</sub> g	L 1 min. DHE 5 <sub>u</sub> g	L 5 min. DHE 1 <sub>u</sub> g	L 5 min. DHE 5 <sub>u</sub> g
0	$\bar{X}$ 59566 n 9 1SD 5829 1SE 1943	3	$\bar{X}$ 288978 n 18 1SD 67893 1SE 16002	294400 8 97118 34336	292190 10 71337 22558	317651 9 57521 19173	262259 9 37133 12378	260867 4 65357 32678	191973 5 69002 30858	215416 4 24472 12236	115973 5 32397 14488
2	$\bar{X}$ 310507 n 18 1SD 139682 1SE 32923	4	$\bar{X}$ 587011 n 18 1SD 105054 1SE 24762	547190 7 123284 46597	598773 10 147208 46551	640355 9 96418 32139	576303 9 138842 46280	338050 4 71780 35890	160666 5 65274 29191	197422 3 58782 33937	148973 5 38665 17291
3 pre	$\bar{X}$ 489914 n 18 1SD 96588 1SE 22766	7	$\bar{X}$ 681107 n 18 1SD 141398 1SE 33327	728041 8 134972 47719	656360 10 177853 56242	661785 9 141772 47257	775667 9 89631 2877	573017 4 113145 56572	304307 5 113486 50752	515366 4 279690 139845	173106 5 46167 20646
9	$\bar{X}$ 666035 n 16 1SD 139127 1SE 34781	9	$\bar{X}$ 689655 n 6 1SD 164132 1SE 67006	689655 6 164132 67006	756166 10 169246 53520	662222 9 49768 16589	790849 8 56339 19919	610644 3 77303 44631	429747 5 182782 81743	610017 4 305483 152741	42705 5 42705 19098

$\bar{X}$  = mean  
N = number  
1SD = one standard deviation  
1SE = one standard error

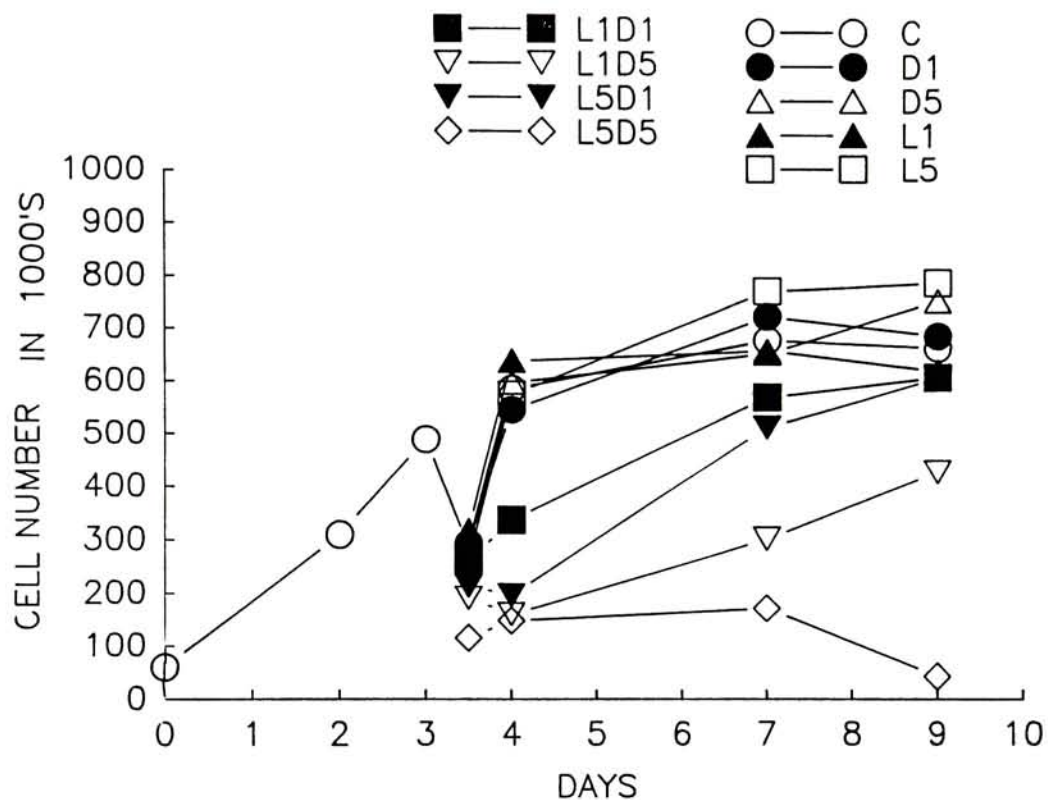


FIGURE 13. Growth curves of pre and post treated groups. C=control, D1=DHE only 1  $\mu$ g/ml, D5=5  $\mu$ g/ml, L1=Light only 1 minute, L5=Light only 5 minutes, L1D1=Light 1 minute and DHE 1  $\mu$ g/ml, L1D5=Light 1 minute and DHE 5  $\mu$ g/ml, L5D1=Light 5 minutes and DHE 1  $\mu$ g/ml, L5D5=Light 5 minutes and DHE 5  $\mu$ g/ml. Day 3.5 is equivalent to 1 hour post irradiation.



## **Quality Control - Cell Counting**

To ensure accurate and precise cell counts, statistics were monitored; counts of a standard solution of 14.7  $\mu$ m latex microspheres, the intra-assay variation of the triplicates, and the level of background counts.

Every Monday during the experiment, the microsphere solution was counted following dilution (1:200). The mean cell count was 5912 (n=10). The intra-assay variation of triplicates was 1.7% (1 CV). The inter-assay or weekly variation was 5.6% (1 CV). The mean intra-assay variation for all cell counts was 2.1% (1 CV).

The background count was measured daily by counting 0.9% saline dilution fluid. The mean background count was  $30.3 \pm 48\%$  (1 CV).

## **Experimental Results**

Cells were plated into wells of a 48 well plate on day 0 at a concentration of  $6 \times 10^4$  cells/ml. At day 2, mean cell count was  $3.1 \times 10^5$  cells/ml and continued to increase to a day 3 pre-treatment level of  $4.9 \times 10^5$  cells/ml.

At one hour following treatment, after two washes, in the DHE only and treatment groups, and two-hour incubation with DHE and the argon laser light treated groups, cell counts dropped to values between  $2.8 \times 10^5$  and  $3.2 \times 10^5$  cells/ml. The decrease of cell numbers in the "control" groups was from washing. Additional loss of attached cells was seen in DHE 5  $\mu\text{g/ml}$  and light treated groups, and in the DHE 1  $\mu\text{g/ml}$  and light-5 minutes group. Cell counts dropped to a level of  $1.2 \times 10^5$  cells.

At 24 hours after treatment, Day 4, the control, DHE only, and light only groups increased to between  $5.5 \times 10^5$  and  $6.4 \times 10^5$  cells/ml and continued to increase slightly until day 9.

The cells treated with DHE-1  $\mu\text{g/ml}$  and light exposure of either one or five minutes recovered at slower rates. At 24 hours post treatment, the light-1 minute, DHE 1  $\mu\text{g/ml}$  cells grew to  $3.4 \times 10^5$  cells/ml, whereas the remaining treated cell groups remained at  $1.6 - 1.9 \times 10^5$  cells/ml.

At 96 hours (Day 7) and 144 hours (Day 9), the cells treated with light and DHE-1  $\mu\text{g/ml}$  continued to

increase in cell number. The group treated with light for one minute and DHE-5  $\mu\text{g/ml}$  showed a slight increase. The cells treated with light for five minutes and DHE-5  $\mu\text{g/ml}$  leveled and then decreased in cell number over the remaining six-day period.

### **Statistical Comparisons of Differences**

The null hypothesis for this experimental design is that the mean cell counts are equal for each group at each sampling time. To test this hypothesis, variance plots were produced from the distribution statistics and test statistics were calculated by analysis of variance (ANOVA) and the Student's t-test.

Variance plots were created and appear in Figures 14A-D, corresponding to one hour (Day 3), 24 hours (Day 4), 96 hours (Day 7), and 144 hours (Day 9) post irradiation.

The variance plots show the mean ( $\bar{X}$ ),  $\pm 1$  and 2 standard errors (horizontal bars),  $\pm 1$  and 2 standard deviations (shaded box bars), and the range (vertical line) of each group.

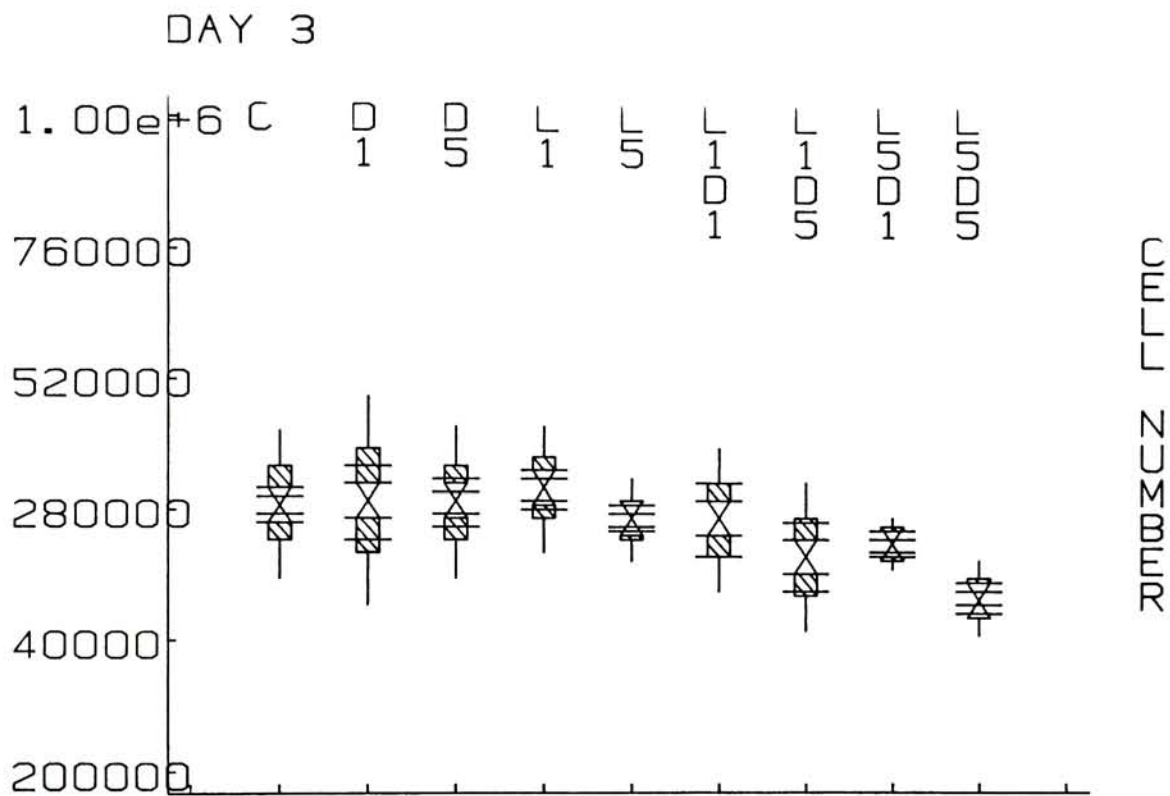


FIGURE 14A. Variance plots of mean cell numbers at 1 hour post irradiation.

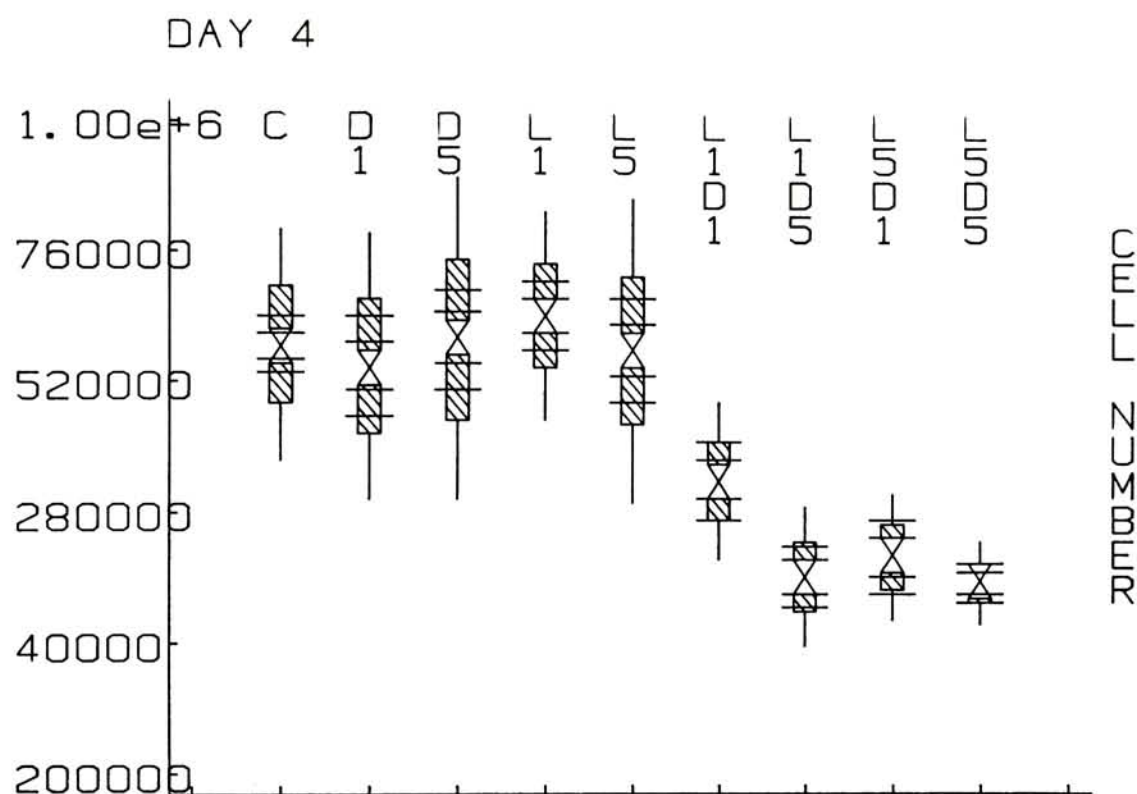


FIGURE 14B. Variance plots of mean cell numbers at 24 hours post irradiation.

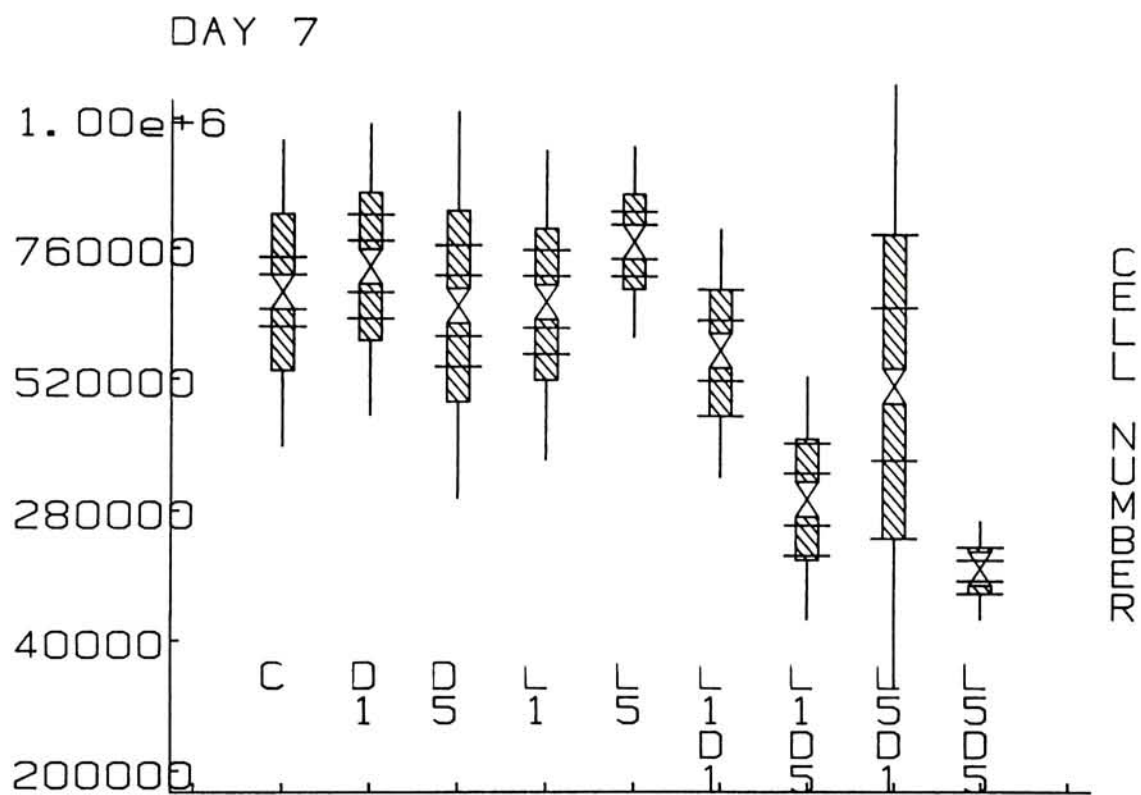


FIGURE 14C. Variance plots of mean cell numbers at 96 hours post irradiation.



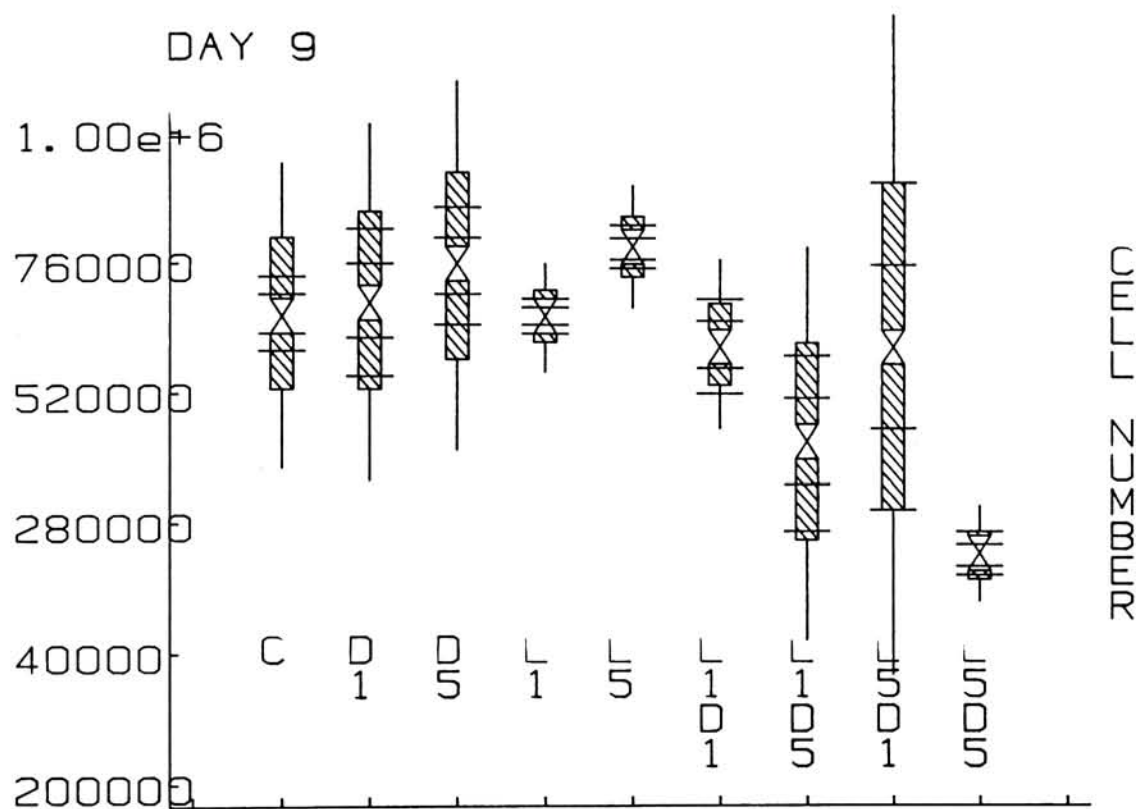


FIGURE 14D. Variance plots of mean cell numbers at 144 hours post irradiation.

These plots allow the visualization of the variance and allow inference of sameness or difference of the means. ANOVA and the t-test give quantitative values of probability of sameness or differences. The results are:

On Day 3 (Figure 14A), at one hour post irradiation, there is no difference (the null cannot be rejected) in the mean cell number of any group except that of the light-5 minutes and DHE-5  $\mu\text{g/ml}$  treated group. The cell number is significantly ( $p < 0.0005$ ) lower than that of all other groups.

Over time, effects of disrupted structure and biochemistry increase. At 24 hours (Day 4), cell numbers decreased in the post irradiation (Figure 14B). Cell numbers decreased in the light and DHE treated groups. There is no difference in the mean cell numbers of the untreated-control, DHE only, and light only groups.

There is no difference in the mean cell numbers of the light and DHE treated groups at light exposures of five minutes and DHE concentrations of 1 and 5  $\mu\text{g/ml}$ . There is a significant difference ( $p < 0.005$ ) of the mean cell numbers of these and of the "control" groups. Light and DHE at these levels cause cells to detach, which indicates cell death.

The light-1 minute and DHE-1  $\mu\text{g/ml}$  treated cells have a mean cell count that is significantly different ( $p < 0.02$ ) from all other treatment groups. At 96 hours (Day 7) post irradiation (Figure 14C), there is no difference in the mean cell numbers of the "control" groups and light-1 minute, DHE 1  $\mu\text{g/ml}$  group (L1D1). There is no difference in the mean cell number of any of the remaining treatment groups.

There is a significant difference ( $p < 0.036$ ) between the mean cell numbers of the "control" groups, L1D1, and the other treatment groups.

At 144 hours (Day 9) post irradiation (Figure 14D), only the light-5 minutes and DHE 5  $\mu\text{g/ml}$  group has significantly ( $p < 0.0002$ ) lower mean cell count, as compared with the other groups.

### **Cell Viability**

Trypan blue dye exclusion test was performed to determine the number of dead cells attached and of all cells--attached and detached--in the sample wells. Live cells exclude the dye. The percent viability was calculated by subtracting the number dead from the total number of cells counted and then multiplying the result by 100.

Photomicrographs of cells in two separate fields were recorded. Cell counts of live and dead cells were recorded. Total number and the number of living cells was calculated. The count of two fields was averaged.

Representative photomicrographs of live and dead (blue) cells at one hour post irradiation appeared in Figures 15A-F. In this underexposed photograph (Figure 15A), control cells were unstained, corresponding to 90+% viability. The same was true for DHE only-5  $\mu\text{g/ml}$  (Figure 15B), light only-1 minute (Figure 15C), and light only-5 minutes (Figure 15D).

Large numbers of trypan blue stained-dead cells were present in the light and DHE treated groups (Figures 15E, 15F).

Mean percent viability, along with other distribution statistics, was calculated for each group at each time period. Results appear in Table 6 and are displayed graphically in Figure 16. Over time, mean viability of cells had a similar trend as the mean cell numbers (growth, Figure 13).



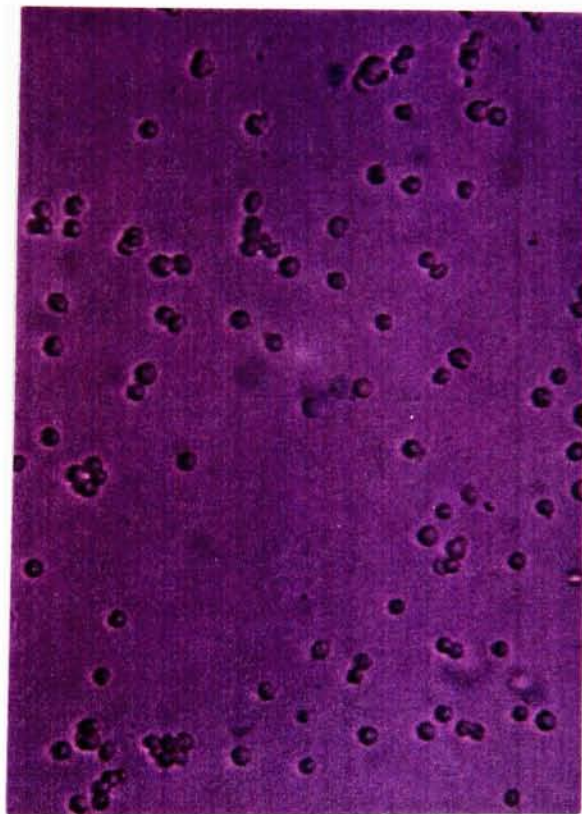


FIGURE 15A. Control cells, 1 hour post treatment. Day 3.

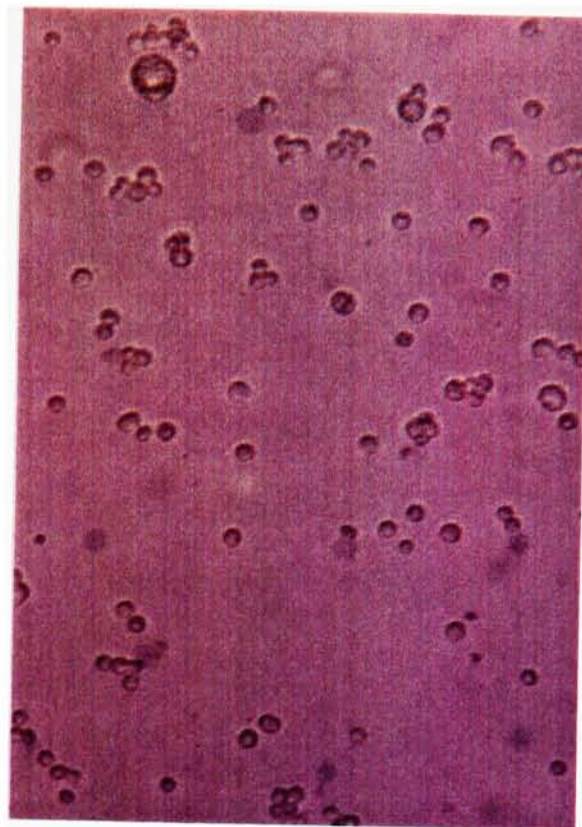


FIGURE 15B. DHE only (5  $\mu$ g/ml) cells 1 hour post treatment. Day 3.





FIGURE 15C. Light only (1 minute)  
cells post treatment.  
Day 3.

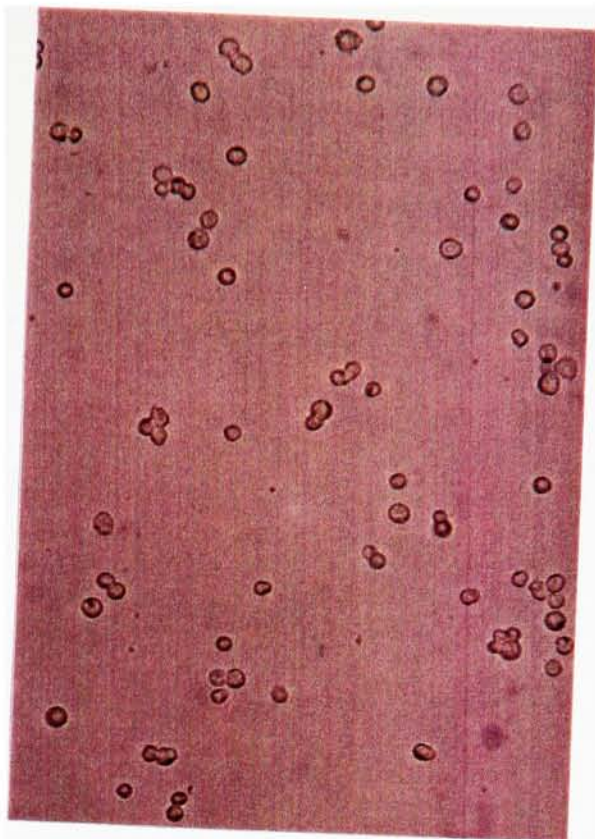


FIGURE 15D. Light only (5 minutes)  
cells 1 hour post treatment.  
Day 3.

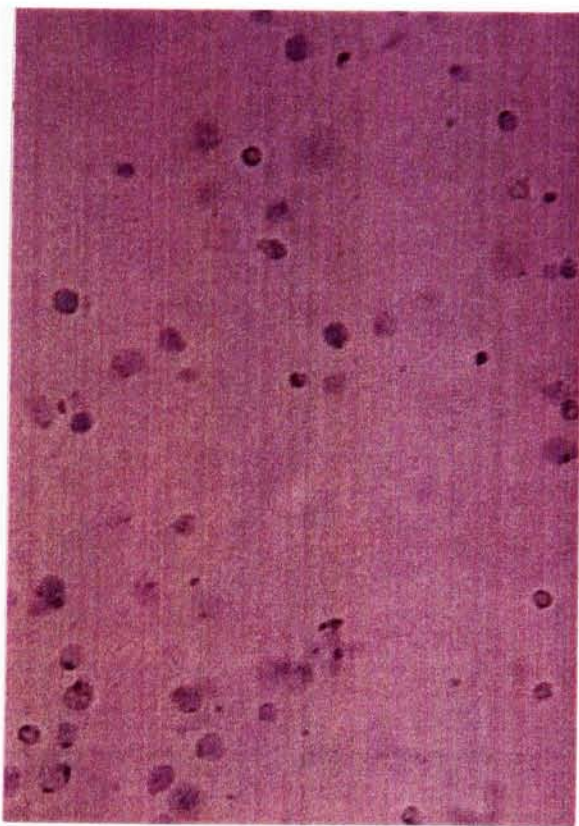


FIGURE 15E. Light (1 minute) and DHE (5  $\mu$ g/ml) cells, 1 hour post treatment. Day 3.

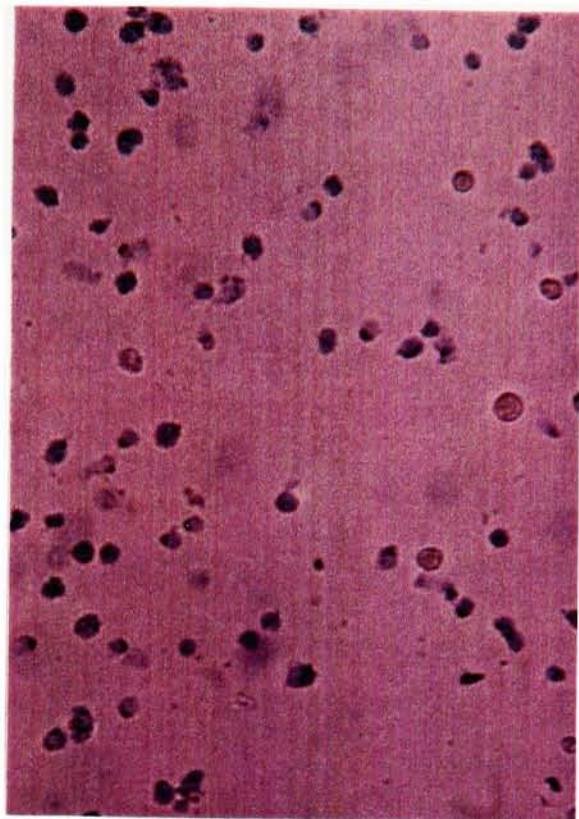


FIGURE 15F. Light (5 minutes) and DHE (5  $\mu$ g/ml) cells, 1 hour post treatment. Day 3.

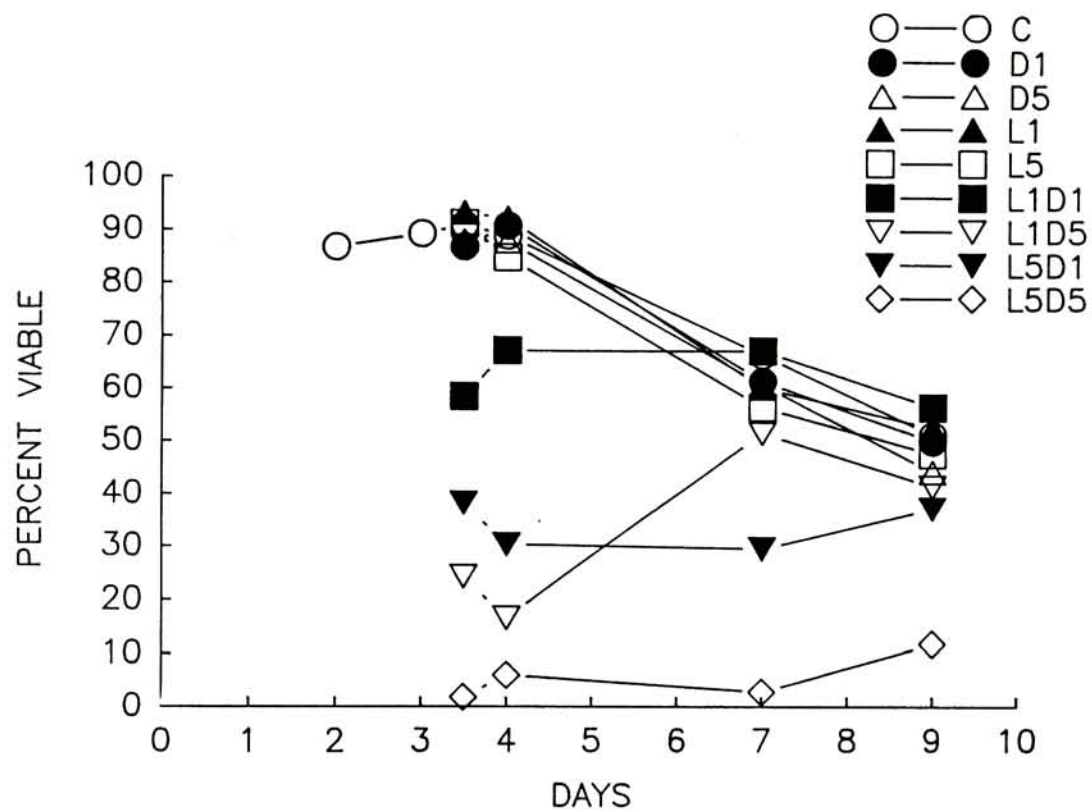


FIGURE 16. Viability of attached cells versus days.

TABLE 6

Cell Viability (%) of Attached Cells - Statistical Data  
of Pre Treatment Control Wells

Day	Control	Day	Control	DHE (1 g/ml)	DHE (5 g/ml)	Light 1 min.	Light 5 min.	L 1 min. DHE 1 g	L 1 min. DHE 5 g	L 5 min. DHE 1 g	L 5 min. DHE 5 g
2	$\bar{X}$ 86.9 N 15 1SD 8.1 1SE 2.1	3	$\bar{X}$ 91.2 N 17 1SD 6.6 1SE 1.6	87.1 8 14.2 5.0	88.2 10 7.4 2.3	93.5 8 3.0 1.1	91.5 9 7.1 2.3	58.7 4 25.5 12.7	24.4 5 20.0 8.9	38.4 4 24.6 12.3	1.8 5 3.4 1.5
3AC	$\bar{X}$ 89.5 N 14 1SD 6.8 1SE 1.8	4	$\bar{X}$ 89.2 N 18 1SD 7.2 1SE 1.7	91.1 8 5.8 2.1	88.1 10 5.9 1.8	92.5 9 5.4 1.8	85.1 9 15.4 5.1	67.5 4 31.5 15.8	16.7 5 17 7.6	30.5 3 7.3 4.2	6.0 5 6.6 2.9
		7	$\bar{X}$ 66.9 N 18 1SD 18.5 1SE 4.4	61.9 8 25.4 9.0	61.2 10 18.4 5.8	60.6 9 15 5.0	56.9 9 18 6	67.7 4 23.7 11.8	51.9 5 11.9 5	29.8 3 25.1 15.5	2.8 5 4.9 2.2
		9	$\bar{X}$ 51.7 N 16.0 1SD 20.0 1SE 5.0	50.4 6 20.8 8.5	44.6 10 10.5 3.3	53 9 17.8 5.9	48 9 15.2 5.1	57 3 28.9 16.7	41.8 5 16.9 7.5	37.6 4 15.9 8.0	12 5 17.7 7.9

$\bar{X}$  = mean

N = number

1SD = one standard deviation

1SE = one standard error



Pre-treatment, mean cell viability was 86.9% on Day 2 post plating, and 89.5% on Day 3. At one hour post treatment and irradiation, mean cell viability was between 87.1% and 93.5% in the untreated, DHE only, and light only groups. The mean cell viability decreased and showed values from 58.7% to 1.8% with increasing light (1 to 5 minutes) and increasing DHE (1 to 5  $\mu\text{g/ml}$ ).

At 24 hours (Day 4), the mean cell viability of the "control" groups was between 85.1% for light only at 5 minutes to 92.5% for light only at 1 minute. The light-1 minute and DHE-1  $\mu\text{g/ml}$  group showed a mean viability of 67.5% which was increased as compared to Day 3, 1 hour post irradiation. This indicates that the cells were increasing their numbers of live cells.

The mean viability of the light-1 minute and DHE-1, 5  $\mu\text{g/ml}$  treated groups decreased as compared to Day 3, 1 hour post irradiation and were significantly lower than other groups. This is due to cells that were still dying. The light-5 minutes and DHE-5  $\mu\text{g}$  mean cell viability increased slightly.

At 96 hours (Day 7), there was an overall decrease in mean cell viability. The cell medium was running out



of nutrients along with the build up of toxic and inhibitory substances. The exception being the light-1 minute DHE-5  $\mu\text{g/ml}$  which increased.

Finally, at 144 hours (Day 9) post irradiation, mean cell viability continued to decrease in all groups, except in the light-5 minutes, DHE-1 and 5  $\mu\text{g/ml}$  groups.

Plots (Figures 17A-D) were created to show variance and help explain differences between the mean cell viability of each group, at each time period post irradiation.

A comparison of differences of means was determined by ANOVA and Student's t-test. The null hypothesis is that the means are equal. For Day 3, one hour post irradiation, there is no significant difference between the control groups; in other words, the means are equal.

Light and DHE alone do not affect cell viability. There is no significant difference ( $p > 0.05$ ) between "control" groups and light at 1 and 5 minutes, DHE 1  $\mu\text{g/ml}$  groups. There is a significant difference ( $p < 0.05$ ) between the "control" groups and light 5 minutes, DHE 1  $\mu\text{g/ml}$ , and light-1 and 5 minutes, DHE 5  $\mu\text{g/ml}$  groups.

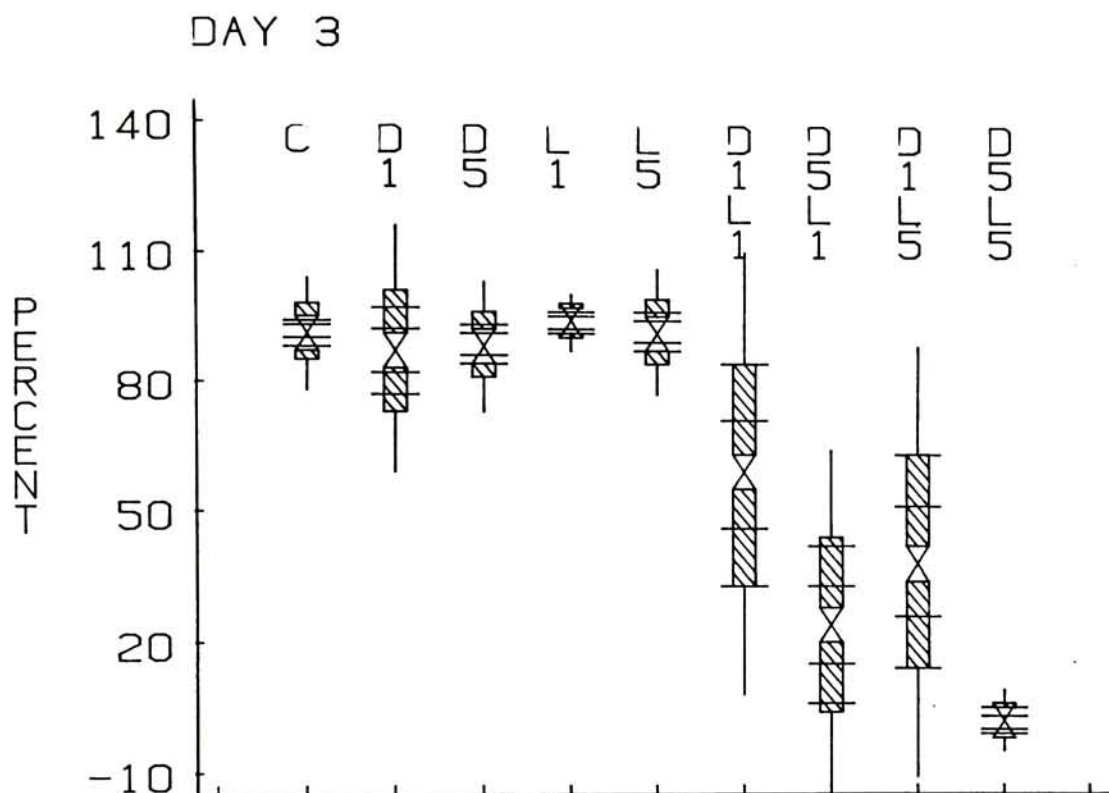


FIGURE 17A. Variance plot of mean viability (%) of attached cells, one hour post irradiation (Day 3). [(X) - mean, (-) - 1 and 2 SE, (bar) - 1 and 2 SD, (|) - range].

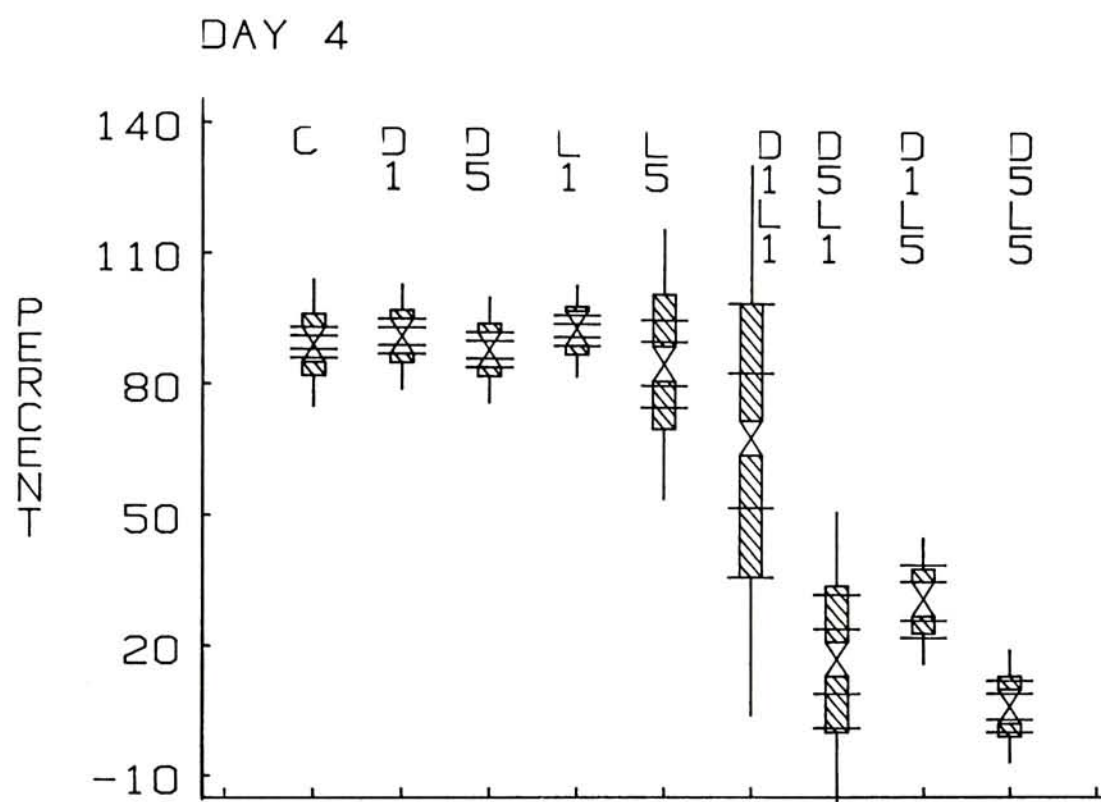


FIGURE 17B. Variance plot of mean viability (%) of attached cells, 24 hours post irradiation (Day 4). [(X) - mean, (-) - 1 and 2 SE, (bar) - 1 and 2 SD, (|) - range].

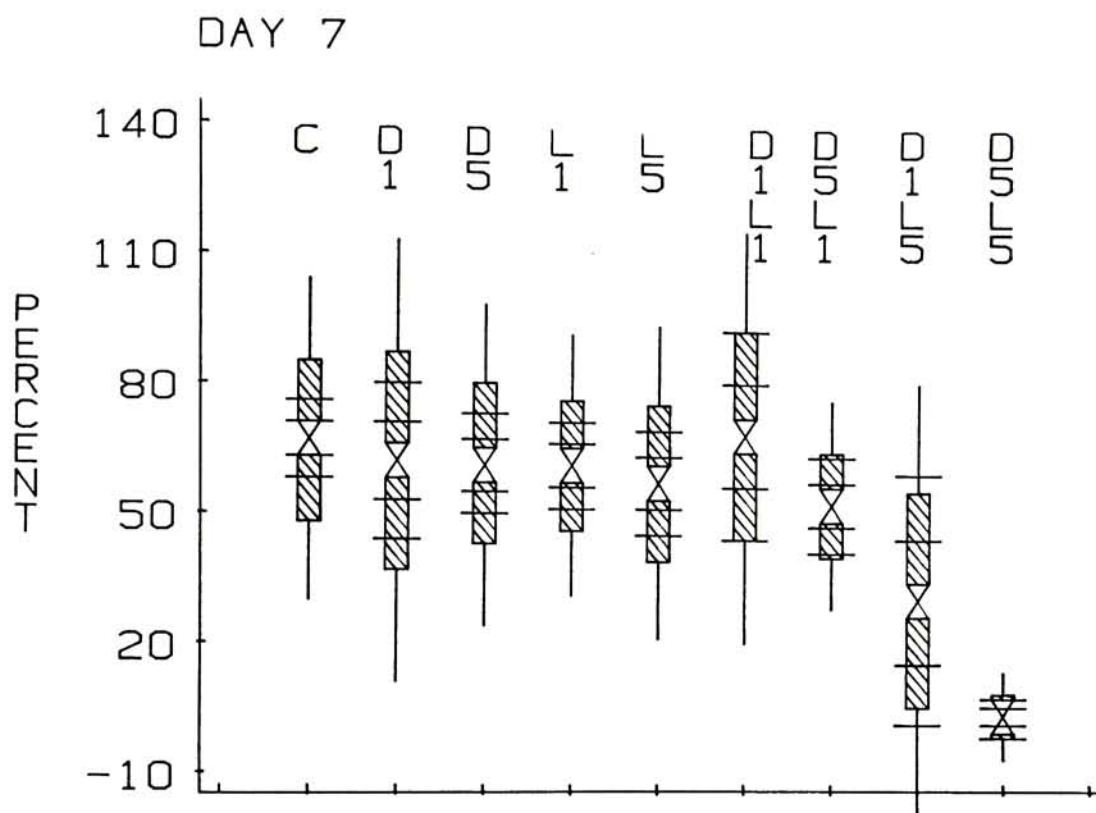


FIGURE 17C. Variance plot of mean viability (%) of attached cells, 96 hours post irradiation (Day 7). [(X) - mean, (-) - 1 and 2 SE, (bar) - 1 and 2 SD, (|) - range].

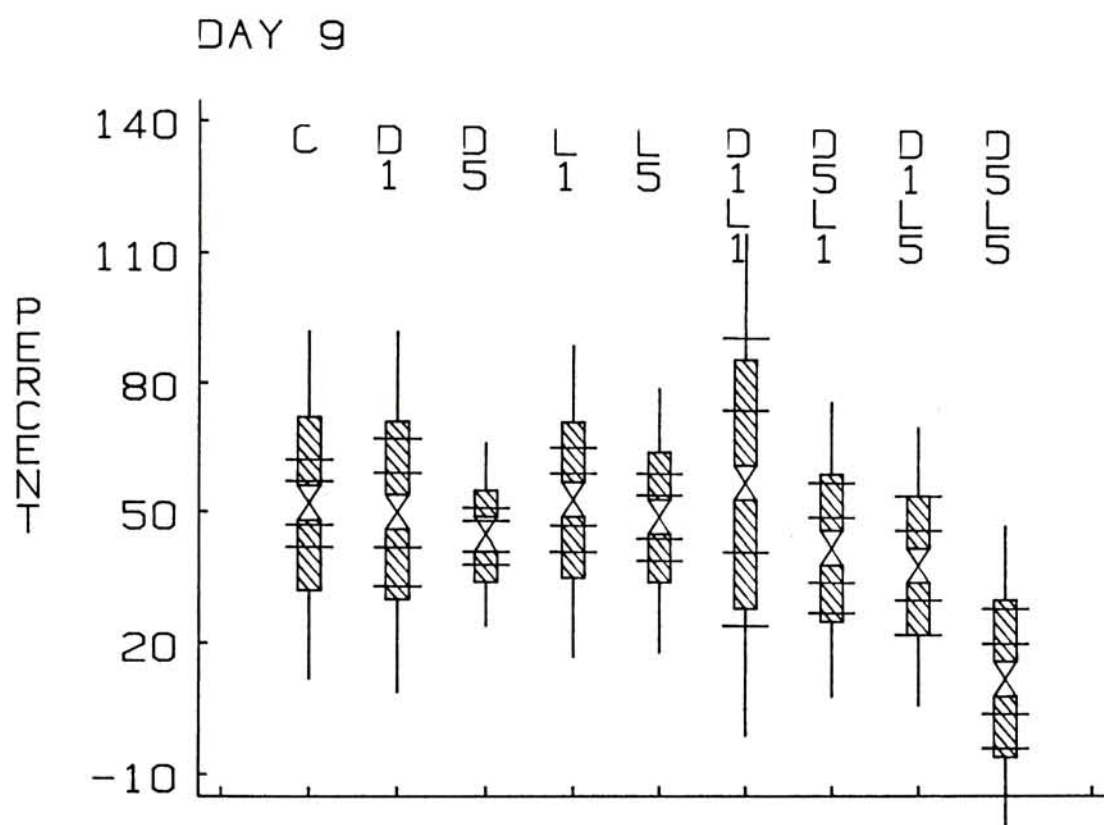


FIGURE 17D. Variance plot of mean viability (%) of attached cells, 144 hours post irradiation (Day 9). [(X) - mean, (-) - 1 and 2 SE, (bar) - 1 and 2 SD, (|) - range].



At 24 hours post irradiation (Day 4), mean cell viability of all "control" groups was the same, as were all the "treatment" groups. There was a significant difference ( $p < 0.001$ ) between the mean cell viability of the "control" groups and of the treatment groups.

At 96 hours (Day 7) and 144 hours (Day 9) post irradiation, there was no difference in the mean viability except with the light-5 minutes, DHE-5  $\mu\text{g/ml}$  group [Day 7,  $p < 0.0001$ ; Day 9,  $p < 0.05$ ].

#### **Viability - Attached and Detached Cells**

An experiment was carried out to determine the cell viability in the wells of both attached and detached cells. When cells were killed, they detached from the tissue culture plate surface and floated in the medium. Since the medium was aspirated and saved for catecholamine analysis, the cell viability of the "floaters" was unknown.

The results of the experiment were tabulated in Table 7 and are shown in Figure 18. Variance plots were constructed and appear in Figures 19A and 19B.

**TABLE 7**

Viability (% , mean  $\pm$  1 CV) of Attached and Detached Cells at 1 and 24 Hours Post Treatment

<u>Group</u>	<u>1 Hour</u>	<u>24 Hours</u>
C*	86	92
L1	96 $\pm$ 0.01	94 $\pm$ 0.04
L5	96 $\pm$ 0.02	94
L1D1	94 $\pm$ 0.01	84 $\pm$ 0.09
L5D1	86 $\pm$ 0.03	87 $\pm$ 0.02
D5*	89	90
L1D5	48 $\pm$ 0.04	14 $\pm$ 0.39
L5D5	4 $\pm$ 0.82	9 $\pm$ 0.25

L = Light for 1 or 5 minutes

D = DHE concentration, 1 or 5  $\mu$ g/ml

\*n = 1, all others n = 4

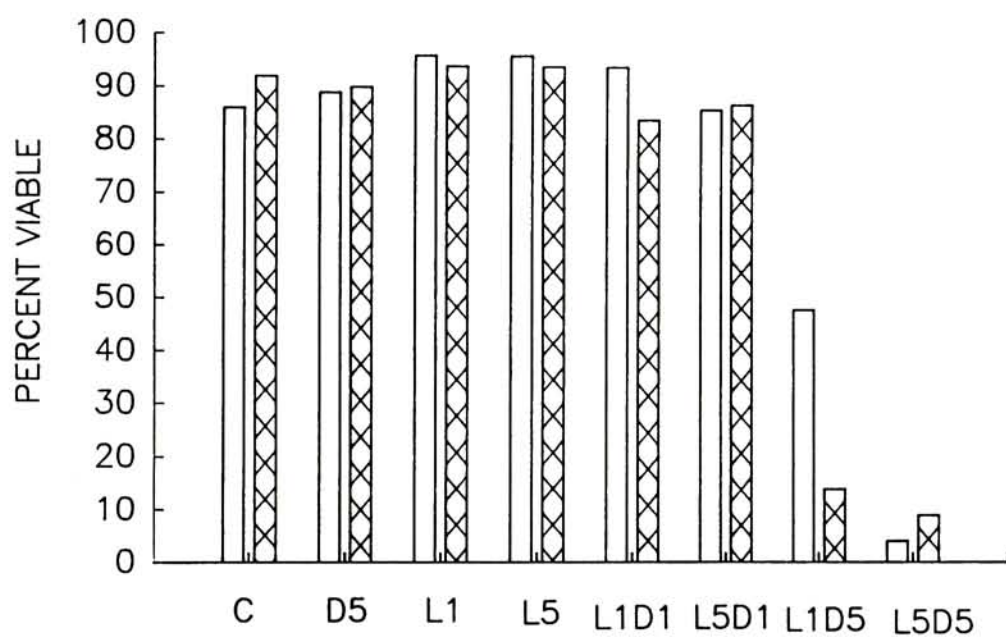


FIGURE 18. Mean viability (%) of attached and detached cells, 1 and 24 hours post treatment.

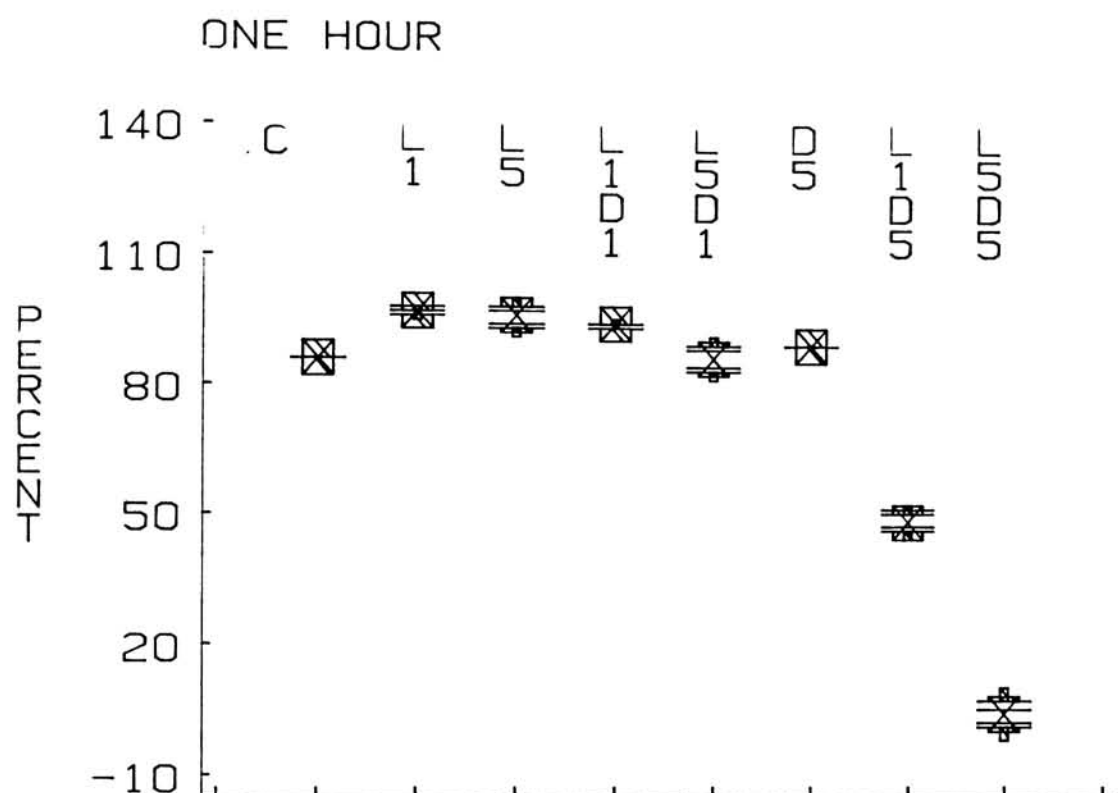


FIGURE 19A. Viability of attached and detached cells at 1 hour post irradiation.

[(X) - mean, (-) -  $\pm 1$  and 2 SE, (bar) -  $\pm 1$  SD, (|) - range).

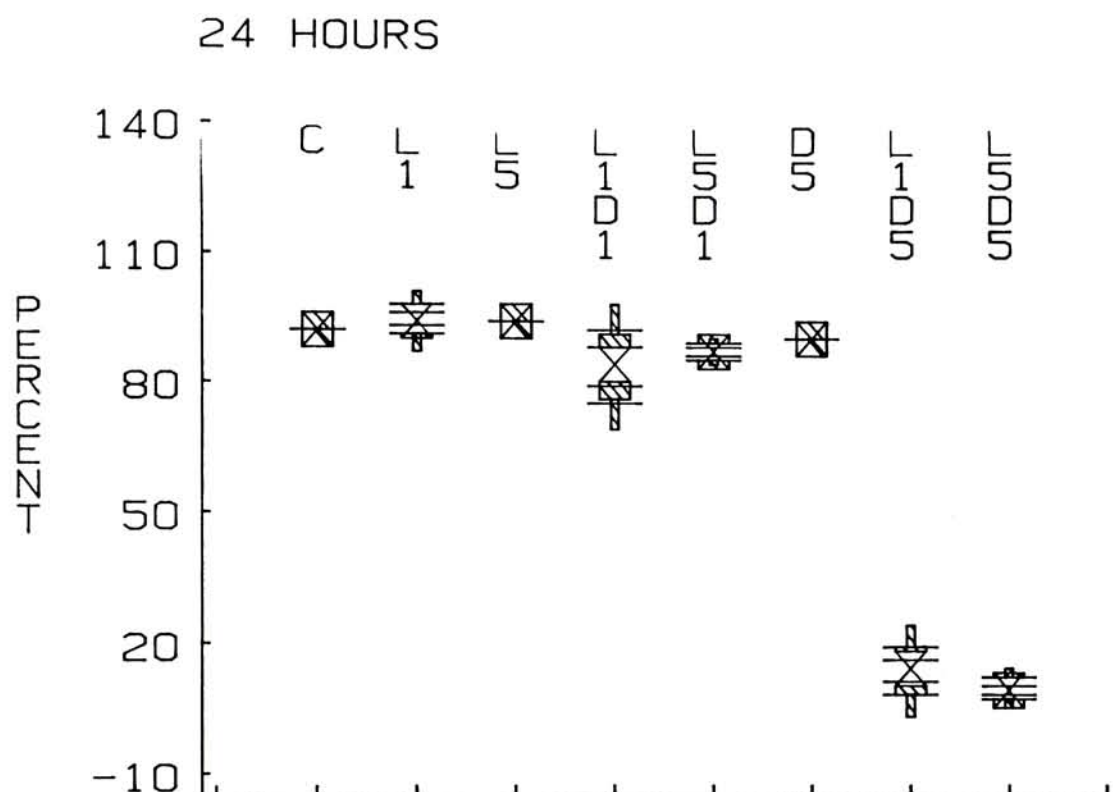


FIGURE 19B. Viability of attached and detached cells, 24 hours post irradiation.  
 [(X) - mean, (-) -  $\pm 1$  and 2 SE, (bar)  $\pm 1$  SD, (|) - range).



**TABLE 8**

Comparison of Viability of Attached Only  
and All Cells - Attached and Detached

	1 Hour		24 Hours	
	Attached $\bar{X} \pm 1SD$	All $\bar{X} \pm 1SD$	Attached $\bar{X} \pm 1SD$	All $\bar{X} \pm 1SD$
C	91 $\pm 6.6$	86	89 $\pm 7.2$	92
D1	87 $\pm 14.0$	ND*	91.1 $\pm 5.8$	ND
D5	88 $\pm 7.4$	89	88.1 $\pm 5.9$	90
L1	93.5 $\pm 3.0$	96 $\pm 1.0$	92.5 $\pm 5.4$	94 $\pm 3.8$
L5	91.5 $\pm 7.1$	96 $\pm 1.9$	85.1 $\pm 15.4$	94 $\pm 0.0$
L1D1	**58.7 $\pm 25.5$	94 $\pm 0.9$	67.5 $\pm 31.5$	84 $\pm 7.6$
L5D1	**38.4 $\pm 24.6$	86 $\pm 2.6$	**30.5 $\pm 7.3$	87 $\pm 1.7$
L1D5	***24.4 $\pm 20.0$	48 $\pm 1.9$	16.7 $\pm 17.0$	14 $\pm 5.5$
L5D5	1.8 $\pm 3.4$	4 $\pm 3.3$	6.0 $\pm 6.6$	9 $\pm 2.2$

\*ND - not determined  
\*\* difference  
\*\*\* slight difference

At one hour post, the cell viability was 86% for the control group. The mean cell viability at one hour post irradiation was between 86% and 96% for all groups except for L1D5 and L5D5, which are 48% and 4%. The differences between these and other groups are evident.

At 24 hours post irradiation, the cell viability of the control group was 92%. The mean cell viability for all groups except L1D5 and L5D5 was between 84% and 94%. The mean cell viability for L1D5 and L5D5 is 14% and 9%, respectively. The difference between these groups and the others is evident.

Differences in mean cell viability occurred only in L1D1, L5D1, and L1D5 at one hour post irradiation and in L5D1 at 24 hours. This means that in these groups, cells that were detached were not dead as determined by trypan blue dye exclusion.

A comparison of these results to the viability of only attached cells is shown in Table 8 (page 91) only. There was no difference in the viability except between groups L1D1, L5D1, and L1D5 at one hour.

## **Catecholamine Analysis**

Catecholamine--dopamine, epinephrine, and norepinephrine--levels were measured in tissue culture medium removed from wells containing growing C1300 MNB cells. The differential catecholamine Cat-A-Kit<sup>R</sup> assay system was used for all determinations. Each kit assayed 22 samples, including normal human plasma control.

Seventy samples of medium were assayed using ten kits. Samples were from two replicates, one that used a DHE concentration of 1  $\mu\text{g/ml}$  and one that used a DHE concentration of 5  $\mu\text{g/ml}$ . Samples from all wells and all groups were included from each replicate.

## **Quality Control**

Accuracy and precision of the catecholamine analysis was determined by measuring the concentration of human plasma control provided in the test kit. Since the assay has been developed as a kit, it was expected that the determinations were able to be accurately and precisely reproduced.

All samples were run in duplicate. The mean variation ( $\pm 1$  CV) of duplicates for all samples analyzed was for dopamine (DA), 15.9%; epinephrine (E), 11.7%; and norepinephrine (NE), 13.1%.

Amersham, the Cat-A-Kit<sup>R</sup> manufacturer, reports that the assay is linear between 2-20 and 3000 pg for a 50  $\mu$ l sample. A check on linearity of the assay was performed by analyzing human plasma control at dilutions of 1:1, 1:5, and 1:10. Only two dilutions were made because of the need to limit samples due to cost and time. Two determinations were made at each dilution. The mean value was used for comparison. Results appear in table 9 and in Figures 20 and 21. To verify true linearity, serial dilutions (more than three) should be made over the range of concentrations expected.

Referring to Table 9, one can see a comparison of the measured and expected values. The values matched at dilutions of 1:5 and 1:10 for epinephrine and norepinephrine, but are low for dopamine. At 1:1, the measured result is the same as the expected result.

The line plots in Figure 20 show that the determination appears to be linear between 54.5 and 700.3 pg/ml

TABLE 9

Linearity of Cat-A-Kit<sup>R</sup> Assay  
Using Human Plasma Control  
DA - Dopamine, E - Epinephrine, NE - Norepinephrine

Serial Dilution	Measured DA	Expected DA	Measured E	Expected E	Measured NE	Expected NE
1:1	633	633	700	700	771	771
1:5	22	127	125	140	136	154
1:10	0	63	54	70	78	77



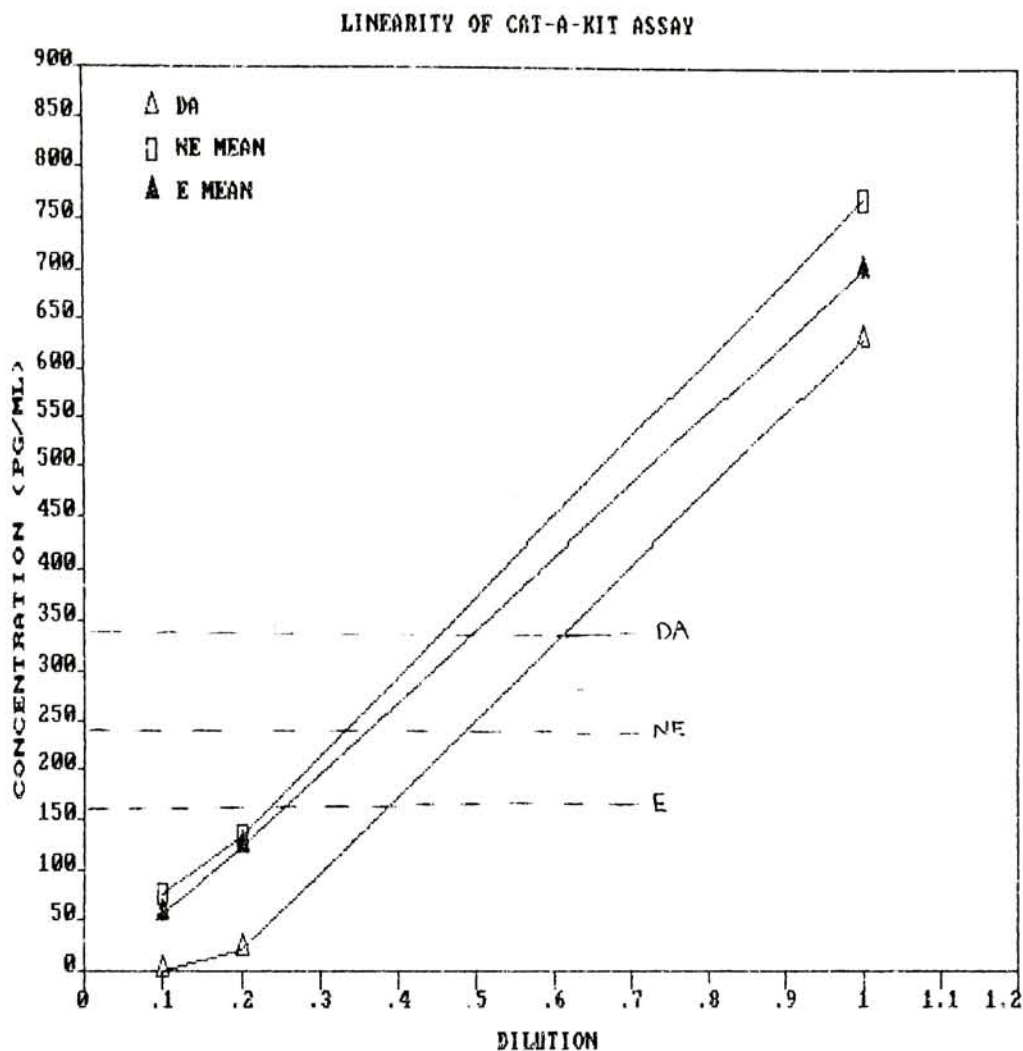


FIGURE 20. Linearity of Cat-A-Kit<sup>R</sup> assay. Serial dilutions (1:5, 1:10) of human plasma control each point for NE and E represent the mean of 2 points. DA single point. Dashed lines show assay detection limit as calculated by 2x background counts of blank.

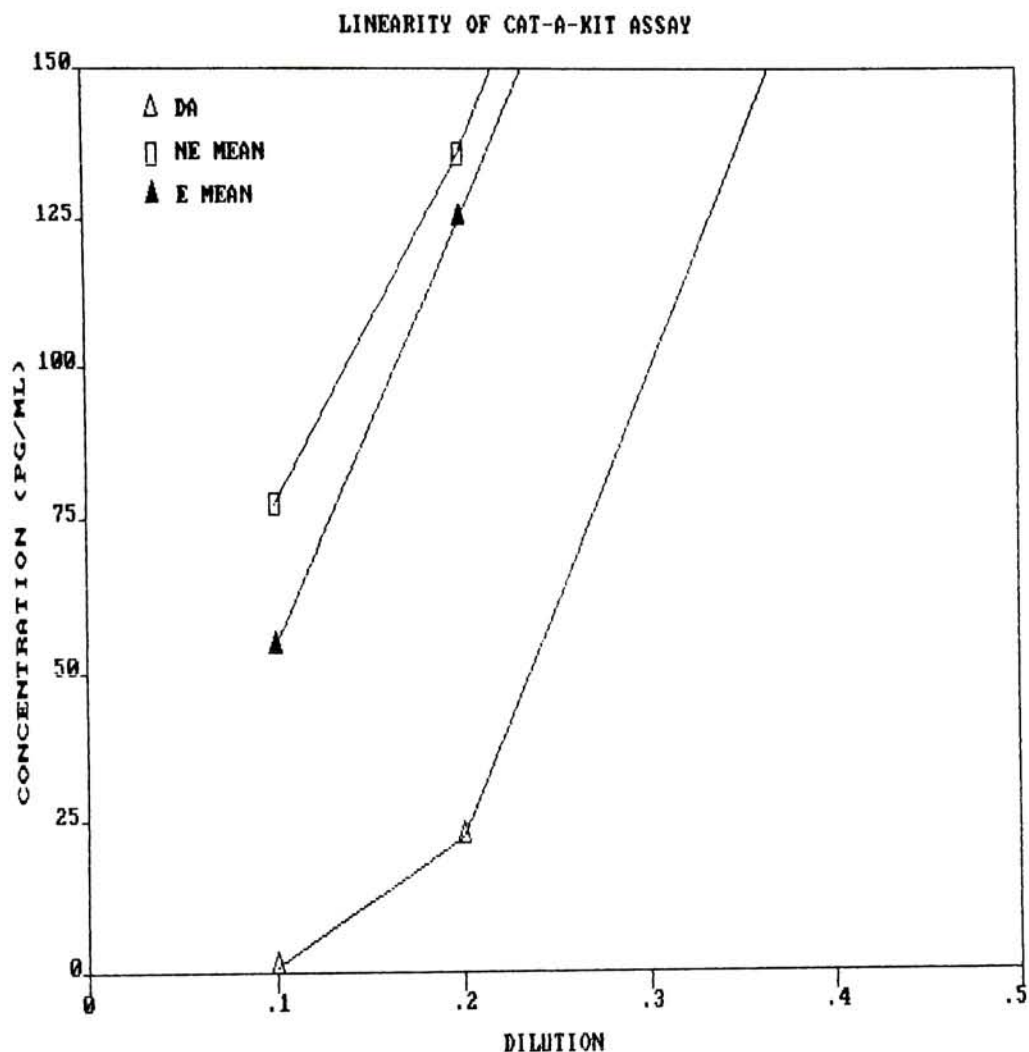


FIGURE 21. Linearity of Cat-A-Kit<sup>R</sup> assay. Expansion of range of graph in Figure 20 (0-150 pg/ml). All values below in house assay detection limit as calculated by 2x background counts of blank.

for epinephrine and 77.7 and 770.7 pg/ml for norepinephrine. The plot for dopamine is not linear because low values are obtained at low concentrations (sensitivity and detection limit). An expanded scale of 0 to 150 pg/ml appears in Figure 21.

Amersham reports that the assay is sensitive to twice the radioactivity of the sample blanks. They report values of 1.2 pg NE/cpm blank, 0.9 pg E/cpm blank, and 7.1 pg DA/cpm blank (cpm - counts per minute). The realized assay detection limit (2x radioactivity of blank) is NE 2.4 pg/0.05 ml (2-5 pg/0.05 ml), E 1.8 pg/0.05 ml (2-5pg/0.05 ml), and DA 14.2 pg/0.05 ml (15-20 pg/0.05 ml). This means that the assay is sensitive to levels of 40-100 pg/ml of E and NE and 300-400 pg/ml of dopamine.

Experimental sensitivity was 0.67 pg/cpm DA, 0.83 pg/cpm E, and 1.08 pg/cpm NE. Sample blanks had cpm's of 225 for DA, 92 for E, and 106 for NE. The realized calculated experiment assay detection limit is 335 pg/ml for DA, 153 pg/ml E, and 228 pg/ml for NE.

Although results of the diluted human plasma control show detection to approximately 70 pg/ml for E

and NE, this is closer to the limit determined from pg/Blank 1 x cpm, that were equal to 61.6 pg/ml for E, 114.5 pg/ml for NE, and 150 pg/ml of DA.

Accuracy of the assay was determined by measuring concentration in human plasma control and comparing the results to values given by Amersham. These results appear in Table 10. Two lots of the assay kit were used. The values +150 match.

It is important to show agreement between replicate measurements. Precision was determined for Cat-A-Kit<sup>R</sup> assay. Inter-assay variation between runs and between kits for human plasma control appears in Table 11. Pueller developed the single isotope radioenzymatic assay. His variation is reported as well as that of Amersham.

The experimental variation (± CV) between run, combined lots, was 13.4% for NE, 10.8% for E, and 10.1% for DA. These are the same as Amersham's and Pueller's measured variation.

The experimental between kit variation (± CV) was 11.2% for NE, 6.4% for E, and 7.7% for DA. The variation

TABLE 10

Accuracy of Cat-A-Kit<sup>R</sup>  
 Catecholamines (pg/ml) in Human Plasma Control  
 $\bar{x} \pm CV$

	NE pg/ml	E pg/ml	DA pg/ml
Amersham			
Lot 7A	650 $\pm$ 91	600 $\pm$ 102	640 $\pm$ 116
Lot 8A	680 $\pm$ 95	700 $\pm$ 119	750 $\pm$ 135
Experimental			
Lot 7A	554 $\pm$ 11	596 $\pm$ 33	552 $\pm$ 20
Lot 8A	697 $\pm$ 100	687 $\pm$ 113	658 $\pm$ 72



**TABLE 11**

Precision of Cat-A-Kit<sup>R</sup>  
 Inter-Assay Variation (CV) Between-Run and Between Kit  
 of Human Plasma Control

<b>Between-Run</b>		<u>DA</u>	<u>E</u>	<u>NE</u>
Pueller		15.5	9.4	10.3
Amersham		12.6	13.5	7.8
Experimental	Lot 7A (n=2)	3.6	5.6	11.0
	Lot 8A (n=7)	7.7	9.6	10.9
	Combined	10.1	10.8	13.4
<b>Between Kit</b>				
Pueller		15.5	9.4	10.3
Amersham		12.6	13.5	7.8
Experimental	(n=5)	7.7	6.4	11.2

for NE was the same as obtained by Pueller and greater than Amersham's. The experimental variation was less for E and DA than obtained by Pueller and Amersham.

Within-run variation was not determined due to limited number sample runs per kits. Historically, Pueller and Amersham report variation ( $\pm$  CV) between 2.7 and 4.6% (Table 12).

The Cat-A-Kit<sup>R</sup> assay is sensitive and accurate with acceptable (10%) precision.

### **Experimental Results**

Results of differential catecholamine analyses of tissue culture media appear in Tables 13-19. Medium over cells from individual wells was sampled. One sample per well was analyzed for catecholamines. The results of the pre-treatment concentrations of DA, E, and NE at 0, 2, and 3 days post plating are shown in Table 13.

Remembering that experimental detection limits (pg/ml) for catecholamines were 335 pg/ml for DA, 153 pg/ml for E, and 228 pg/ml for NE, the results were reviewed.

**TABLE 12**

Precision of Cat-A-Kit<sup>R</sup>  
Historical Within-Run Variation (CV)

	DA	E	NE
Pueller (n=6)	2.7	3.1	3.5
Kit	4.6	3.6	4.2
Experimental	ND*	ND	ND

\*Not Determined

**TABLE 13**

Pre-treatment Concentration† of Dopamine, Epinephrine,  
and Norepinephrine in Media

1 µg/DHE/ml Day	Dopamine pg/ml	Epinephrine pg/ml	Norepinephrine pg/ml
0*	17	0	5
2	130	16	0
3	35	48	10

5 µg/DHE/ml

0**	17	0	5
2	188	42	22
3	76	0	8

†All results below detection limit of assay

\*Assayed once

\*\*Same as 1 µg value, listed for comparison

**TABLE 14**

Concentration of Dopamine (pg/ml) in Medium  
DHE Concentration - 1  $\mu$ g/ml

Days	C	D1	L1	L5	L1D1	L5D5
3 post	127	31	0	4	0	0
4	66	14	141	7	0	0
7	31	0	42	429†	0	0
9	78	0	40	46	0	0

C = Control

D1 = Drug only, 1 minutes

L1 = Light only, 1 minute

L5 = Light only, 5 minutes

L1D1 = Light 1 minute, DHE 1  $\mu$ g/ml

L5D5 = Light 5 minutes, DHE 5  $\mu$ g/ml

Cells Exposed to 1  $\mu$ g DHE/ml for 2 Hours  
and 30 mW/cm<sup>2</sup> Argon Laser

†Only result above detection limit of assay



**TABLE 15**

Concentration of Dopamine (pg/ml) in Medium  
DHE Concentration - 5  $\mu$ g/ml

Days	C	D5	L1	L5	L1D5	L5D5
3 post	485†	27	183	65	177	44
4	0	14	111	9	0	0
7	0	168	180	156	170	0
9	0	95	60	315†	128	0

C = Control

D5 = Drug only, 5 minutes

L1 = Light only, 1 minute

L5 = Light only, 5 minutes

L1D5 = Light 1 minute, DHE 5  $\mu$ g/ml

L5D5 = Light 5 minutes, DHE 5  $\mu$ g/ml

Cells Exposed to 5<sub>2</sub>  $\mu$ g DHE/ml for 2 Hours  
and 30 mW/cm<sup>2</sup> Argon Laser

†Only results at or above detection

**TABLE 16**

Concentration of Epinephrine (pg/ml) in Medium  
DHE Concentration - 1  $\mu$ g/ml

Days	C	D1	L1	L5	L1D1	L5D1
3 post	42	30	18	23	4	8
4	29	20	18	10	17	0
7	25	0	26	742†	7	5
9	23	41	7	0	0	4

C = Control

D1 = Drug only, 1 minute

L1 = Light only, 1 minute

L5 = Light only, 5 minutes

L1D1 = Light 1 minute, DHE 1  $\mu$ g/ml

L5D1 = Light 5 minutes, DHE 1  $\mu$ g/ml

Cells Exposed to 1<sub>2</sub> $\mu$ g DHE/ml for 2 Hours  
and 30 mW/cm<sup>2</sup> Argon Laser

†Only result above detection limit of assay

TABLE 17

Concentration of Epinephrine (pg/ml) in Medium  
DHE Concentration - 5  $\mu$ g/ml

Days	C	D5	L1	L5	L1D5	L5D5
3 post	0	0	21	29	39	421†
4	0	0	46	22	272†	23
7	0	35	37	16	52	0
9	609†	35	30	28	17	157†

C = Control

D5 = Drug only, 5 minutes

L1 = Light only, 1 minute

L5 = Light only, 5 minutes

L1D5 = Light 1 minute, DHE 5  $\mu$ g/ml

L5D5 = Light 5 minutes, DHE 5  $\mu$ g/ml

Cells Exposed to 5<sub>2</sub> $\mu$ g DHE/ml for 2 Hours  
and 30 mW/cm<sup>2</sup> Argon Laser

†Only results at or above detection limit of assay

**TABLE 18**

Concentration of Norepinephrine (pg/ml) in Medium  
DHE Concentration - 1  $\mu$ g/ml

Days	C	D1	L1	L5	L1D1	L5D1
3 post	506†	392†	678†	535†	337†	444†
4	301†	374†	25	377†	682†	0
7	104	0	423	253†	431†	0
9	10	4	0	0	0	0

C = Control

D5 = Drug only

L1 = Light only, 1 minute

L5 = Light only, 5 minutes

L1D1 = Light 1 minute, DHE 1  $\mu$ g/ml

L5D1 = Light 5 minutes, DHE 1  $\mu$ g/ml

Cells Exposed to 1<sub>2</sub> $\mu$ g DHE/ml for 2 Hours  
and 30 mW/cm<sup>2</sup> Argon Laser

†Only results at or above detection limit of assay

**TABLE 19**

Concentration of Norepinephrine (pg/ml) in Medium  
DHE Concentration - 5  $\mu$ g/ml

Days	C	D5	L1	L5	L1D5	L5D5
3 post	7	0	0	8	5	743†
4	0	0	110	0	148	26
7	0	49	0	26	25	0
9	388†	0	0	8	21	664†

C = Control

D5 = Drug only

L1 = Light only, 1 minute

L5 = Light only, 5 minutes

L1D5 = Light 1 minute, DHE 5  $\mu$ g/ml

L5D5 = Light 5 minutes, DHE 5  $\mu$ g/ml

Cells Exposed to 5<sub>2</sub> $\mu$ g DHE/ml for 2 Hours  
and 30 mW/cm<sup>2</sup> Argon Laser

†Only results above detection limit of assay



Concentration of Dopamine (Tables 14, 15), Epinephrine (Tables 16, 17), and Norepinephrine (Tables 18, 19) are reported by group and post treatment day.

In reviewing the data, most results are below the detection limit. The data above detection limits was variable and no interpretation could be made. The null hypothesis was that the levels of catecholamines in all groups are equal when sampled at the same time. One cannot reject the null hypothesis.

## DISCUSSION

It was expected that the chosen levels of DHE and argon laser light would alter cell metabolism and kill some of the C1300 MNB cells. The robust experimental design measured two concentrations of DHE that were in the range encountered in malignant tissue in vivo, and two doses of argon light that were at levels used for laser induced fluorescence of DHE for detection of malignant tissue.

### Cell Morphology

Changes in cell morphology with both treatment and with time were expected. Structure is related to function.

Exposure with light only-5 minutes caused vacuoles to appear. Damage and death occur from direct damage to DNA (Deoxyribonucleic acid) and other organic molecules (33).

There is photoproduction of toxic compounds in tissue culture media (33) such as the formation of

hydrogen peroxide, intramolecular polymerization, tryptophan is oxidized to N-formylkynurenine and tyrosine is broken into various cleavage products.

In the DHE and light treated groups, pyknotic granules were seen. These are changes in size of golgi apparatus, formation of irregular nuclear membranes, vacuoles (38,39,44,45), and possibly DHE itself.

### **Cell Growth**

In the light and light-DHE treated groups, attached cell numbers decreased because the damaged cytoskeleton (biopolymers) detach from extracellular protein/polymer matrix, thus cells break away from the plate surface. This explains the loss of cells one hour post wash, post irradiation groups. Since cells in these groups are damaged, cell reproduction is impaired or halted. So, with increasing time (growth curve), cell numbers remain the same, increase slightly, or decrease. Cell death with time is because medium is running out of nutrients.

Changes in viability of cells can be explained because of similar effects. It was assumed that all floating cells were dead. This was the case for most

groups--Light-5 minutes, DHE 1  $\mu$ g/ml and Light-1 minute, DHE-5  $\mu$ g/ml at one hour. Some of these cells did not stain with trypan blue, thus are viable. This can be explained by the fact that photodynamic effects take time. Cell machinery has been impaired and biopolymers in the cytoskeleton damaged, but cell membrane has not degraded, yet.

### **Experimental Problems**

Problems were encountered in performing the experiment. Experimental replicates were lost because the laser broke and cultures became contaminated with fungus. This left 9 out of the 14 experiments for data collection and interpretation.

### **Catecholamine Analysis**

The radioenzymatic assay kit for differential catecholamines is a labor intensive, expensive analysis. The analysis requires two days of laboratory manipulations such as incubation, extractions, and centrifugation, and one day for B-counting in a liquid scintillation counter.

The cost and time is why a limited number of samples were analyzed. The cost per sample--reagents, kits, equipment, and labor--was \$110/medium sample.

## Problems with the Assay

The Cat-A-Kit<sup>R</sup> is labor intensive. Most steps are critical. Thus, one must adhere to proper technique in order to yield accurate and precise results. One out of 10 assay runs was rejected due to low counts. Problems associated with running the assay were:

In two runs, there were low counts (CPM) in all samples. This was because of inadequate extraction of derivatives in and out of organic and aqueous layers. This problem was resolved by mixing exactly for the specified time. Systematically low counts were multiplied by a correction factor of yield results in one run.

In one run, failure of the shaking water bath occurred during incubation. This caused a slower rate of the enzymatically controlled transfer of <sup>3</sup>H-SAM to the catecholamines. The samples showed systematically low counts.

Background counts of the blanks were high in two runs. This was due to backwash of radioactive material left in the thin layer chromatography developing tank. More careful and repeated washing with greater volumes of toluene eliminated the radioactive material. The background counts of the blanks dropped by one-half.



## **Interpretation of Catecholamine Results**

Dopamine is present in cytoplasm of cells. Catecholamines, NE and E, are synthesized, stored and taken up into granules at the end of neurite-like extensions.

Any impairment of ATP (adenosine triphosphate) metabolism results with eventual failure of the cell and granule membranes. Light and DHE damages proteins and oxidizes lipids in the membrane.

Catecholamine levels were expected to change in the light-5 minutes and the light and DHE treated groups. Concentration of DA, E, and NE would increase in the cell medium if the cell membranes were ruptured and stored catecholamines were released. The concentration at 1 and 24 hours would be expected to be higher.

Another alternative way that concentration would drop post treatment is if catecholamine metabolism was impaired or membrane proteins damaged inhibiting dopamine-B-hydroxylase--a membrane associated enzyme--function.

Cell growth may have continued with impaired catecholamine metabolism. Results of growth and cell viability suggest that in the Light and DHE treated

groups, both cell reproduction and cell kill occurred. Therefore, it was expected that catecholamine production was also impaired.

Catecholamines could have been oxidized by oxygen in ambient air or photo-oxidized medium constituents. This would have led to a decrease in catecholamine levels with time.

No interpretation can be made since the concentration of catecholamines in medium from independent sample wells were highly variable, and some values were at or below the detection limit of the assay.

### **Experimental Design - Future Work**

Growing and studying cells in tissue culture plates required fastidious techniques to control variables. Time intervals were limited due to the small volume of medium in each well of the 48 well plate. Treating and changing the medium at Day 3 was designed to extend the time interval and maximize cell growth post treatment.

When studying a biochemical, using an in vivo model (i.e. a mouse) would simplify the experiment both in supply of nutrients, control of the experimental variables, and labor.

## CONCLUSION

Argon laser light at the power density of 30 mW/cm<sup>2</sup> and energy densities of 1.8 J/cm<sup>2</sup> and 9.0 J/cm<sup>2</sup> significantly changed cell morphology, numbers, and viability only when used with DHE at 5 µg/ml at 1, 24, 96, and 144 hours post irradiation. The L5D5 group was the most severely affected at all sampling times.

Argon light alone did not alter cell morphology, numbers, or viability, except for morphology at 1 hour post irradiation, when cells were treated with light for 5 minutes (9 J/cm<sup>2</sup>).

Cell media was assayed for the catecholamines dopamine, epinephrine, and norepinephrine. Only a few samples were at or above the detection limit of the Amersham Cat-A-Kit<sup>R</sup> assay. Of these, results were variable. Therefore, no interpretation can be made concerning catecholamine production of C1300 MNB in this experimental model. It could not be concluded from the data that catecholamines would be useful as a specific marker of tumor impairment or death.

At the low energy of levels argon laser light and DHE used for tumor detection, the guarded inference can be made that malignant neuroblastoma cells will be damaged and killed.

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## APPENDIX

1. American Type Culture Collection  
Description of Cells - C1300 MNB - NB41A3
2. Protocol-Tumor Transplant and Culture of C1300 MNB Cells  
in Male AJ Mice
3. Calibration Procedure of Coulter Counter Model Z Calibration Curve Auto vs. Manual Cells
4. Protocol - Fluorescent Catecholamine Staining
5. Protocol - Preparation of Cells for Catecholamines
6. Cat-A-Kit<sup>R</sup> Analysis Assay Protocol
7. Coulochem Applications Notes  
HPCL of Catecholamines
8. Related Publications - Reprints
  - A. Lanzafame, R.J., J.R. Blackman, **D.W. Rogers**, J.O. Naim, R.P. Pennino, J.R. Hinshaw. Hematoporphyrin Derivative Fluorescence: Photographic Techniques for the Localization of Malignant Tissue.
  - B. Lanzafame, R.J., **D.W. Rogers**, J.O. Naim, H.R. Herrera, J.R. Hinshaw. The Effect of Hematoporphyrin Derivative 2 on Estrogen Receptors in the DMBA Mammary Tumor Model.
  - C. McCormack, C.J., J.O. Naim, **D.W. Rogers**, M. Ziegler, J.R. Hinshaw. Effect of CO<sub>2</sub> Laser Excision on Neuroblastoma. Abstract.
  - D. **Rogers, D.W.**, J.R. Blackman, J.O. Naim, R.J. Lanzafame, H.R. Herrera, J.R. Hinshaw. Helium Cadmium Laser-Induced Fluorescence of Dihematoporphyrin Ether for the Endoscopic Detection of Cancer. Abstract.
  - E. Lanzafame, R.J., **D.W. Rogers**, J.O. Naim, H.R. Herrera, J.R. Hinshaw. Photographic Demonstration of Penetration of Dihematoporphyrin Ether Preparations into Tumor Systems. Abstract.



**AMERICAN TYPE CULTURE COLLECTION**  
**12301 Parklawn Drive Rockville, Maryland 20852**

**COLLECTION OF CELL LINES**

**ATCC No.:** CCL 147

**Name:** NB41A3 (Neuroblastoma, C-1300, Mouse)

**Culture Medium:** Ham's F-10 medium, 82.5%; horse serum, 15%; fetal bovine serum, 2.5%.

**Fluid Renewal:** 2-3 x weekly **Subculture Procedure:** Remove medium, add fresh 0.25% viokase for 2-3 minutes, remove and let culture sit for 10-20 minutes at room temperature. Add fresh medium, aspirate, and dispense into new flasks. A subcultivation ratio of 1:2 to 1:4 is recommended.

**Part A. FROZEN CELLS**

**Freeze:** 5924 **Vol./ Ampule:** 1.0 ml **Passage No.:** 46 **Date Frozen:** 1/86 **Cells/ml.:**  $2.7 \times 10^6$

**Expected Viability(%):** 90-95% **Population Doublings:** n/a

**Recommended Handling Upon Receipt:** Initiate culture as soon as possible upon receipt. Thaw by rapid agitation in 37°C water bath. **See instructions on back.**

**Recommended Inoculum Upon Thawing:** 1:12 dilution of ampule contents.

**Part B. FLASK CULTURES**

**No. Cells Seeded Per Culture:**  $2.4 \times 10^6$  **Passage No.:** 47

**Recommended Handling Upon Receipt:**

**Monolayer Cultures:** The bottle is completely filled with medium to prevent loss of cells in transit. Remove all of the medium (which can be saved and used as fresh medium) except for a sufficient volume to cover the floor of the flask. Incubate at 37°C. Sometimes in transit the cultures are handled roughly and most of the cells become detached and float in the culture medium. If this has occurred remove the entire contents of the flask and centrifuge at 300 x g for 15 minutes. Draw off the excess supernatant medium, resuspend the cells in 10 ml of the culture medium and plant the entire cell suspension in a single flask of suitable size (ca 25 sq. cm.).

**Suspension Cultures:** The culture flasks have been completely filled with medium for shipment. Remove the entire contents of the flasks and centrifuge. Resuspend the cell pellet as suggested under subculture procedure described above.

**Part C. REMARKS**

## APPENDIX 2

### PROTOCOL

#### Tumor Transplant/Culture of C-1300 Neuroblastoma Cells in Male A/J Mice

- Purpose:**
1. To enhance growth characteristics of cell line by passing through a host animal.
  2. To establish in vivo tumor model.

**Materials:**

- T-25, 75 flasks
- Ham's F-10 with glutamine with serum
- 50 ml centrifuge tubes, plastic, sterile
- 10, 25 ml serological pipets, sterile
- centrifuge
- hemacytometer
- trypan blue 0.2%
- T.B. syringes
- A/J mice
- C-1300 NB cells, ATCC CCL147 (NB41A3)

#### Methods 1: Tissue Culture (T.C.) to Mouse (C.J. McCormack)

1. Suspend cells in T.C. flask by scraping with rubber policeman.
2. Pipet up and down the total volume of the flask (x5) to mix cells.
3. Centrifuge cells at low G's 5 minutes in T.C. centrifuge tubes (50 ml).
4. Decant supernatant.
5. Resuspend all cells into a single vial, medium Ham's F-10 without serum.
6. Perform cell count.
7. Report total live cells/ml.
8. Centrifuge, resuspend in medium to get  $1 \times 10^6$  cells/0.1 ml.
9. Inject 0.1 cc into mouse.
10. Grow until 2 cm longest, measured every two days.

## A-2.2

### Methods 2: Excision of Tumor for In Vivo or In Vitro Passage

1. Sacrifice mouse by cervical dislocation (ideal tumor size is less than 2 cm longest diameter).
2. Shave area around tumor mass; wipe off loose hair. Dip entire mouse into 70% ethanol or wet totally with 70% ethanol.
3. Wipe down or dip tips of instruments in 95% (or 70%) ETOH and pass through flame to burn off. Excise skin in half-circle around mass. Use scissors to dissect skin off of mass and reflect back the skin flap.
4. Remove non-necrotic chunks of tumor and put into medium (10 cc in petri dish).
5. Press chunks through stainless steel mesh with plunger of 10 cc syringe.
6. Using 10 cc syringe, flush cell suspension and fibrous chunks up and down several times, then draw into syringe and put on 18 g or 21 g needle. Stand on end for 1 minute to settle out chunks.
7. Bend over needle and express cell suspension into centrifuge tube, leaving last 1 cc containing chunks behind.
8. Perform cell count sample and, while counting, spin cells down in centrifuge. Best is 200 to 300 x G for 4 to 5 minutes, but can use 500 x G for 1-1/2 minutes.
9. For injection into mice, resuspend in Ham's F-10 + Pen/Strep, inject proper number of cells in volume of 0.10 cc or 0.05 cc subcutaneously.

For tissue culture, all steps from #3 to #7 should be performed in sterile hood.



### APPENDIX 3

#### Calibration Procedure of COULTER COUNTER Model Z

##### A. Choosing Control Setting

1. Prepare a dilute suspension of the calibration standard (MICROSPHERES 14.71 micrometer dia.); place a beaker of this dilution on the Sample Platform.
2. Open the Control stopcock.
3. Set the threshold dial at 10.
4. Adjust the 1/AMPLIFICATION switch and 1/APERTURE CURRENT switch so that the pulse pattern on the oscilloscope is about one-third the height of the screen (1/AM:4, 1/AP:4).
5. Run a series of five counts. Record each answer. Then find the average (5487.8).
6. Divide this average by two to get the half-count (2743.9). The point on the Threshold Dial where this half-count can be taken will approximate the same point representing the Median Particle Volume (or Diameter).
7. Rotate the Threshold Dial until the threshold appears to coincide with the Median Volume on the screen. Then run a succession of five or more counts, record the answers, and find the average.
8. This average should equal the half-count previously computed. If not, raise or lower the Threshold Dial accordingly. Run five more counts and find the average again. When the half-counts have matched the computed half-counts, the median has been found (Threshold: 37.5).

##### B. Calibration Computation

Calibration constant, K, can now be determined by the formula:

V = average volume of the known system  
I = the 1/APERTURE CURRENT Switch setting  
A = the 1/AMPLIFICATION Switch setting  
T = the setting of Threshold for half-count

With the calibration constant, any unknown volume, V, can be determined for any combination of I, A, or T:  $V = K \cdot I \cdot A \cdot T$



## A-3.2

### Protocol Comparison of Cell Counts - Manual versus Automatic

**Purpose:** To determine accuracy and linear range of cell counting.

**Materials:**

Zeiss microscope  
Hemocytometer  
pasteur pipets  
Coulter counter model ZF  
sample vials  
C1300 MNB cells

**Methods:**

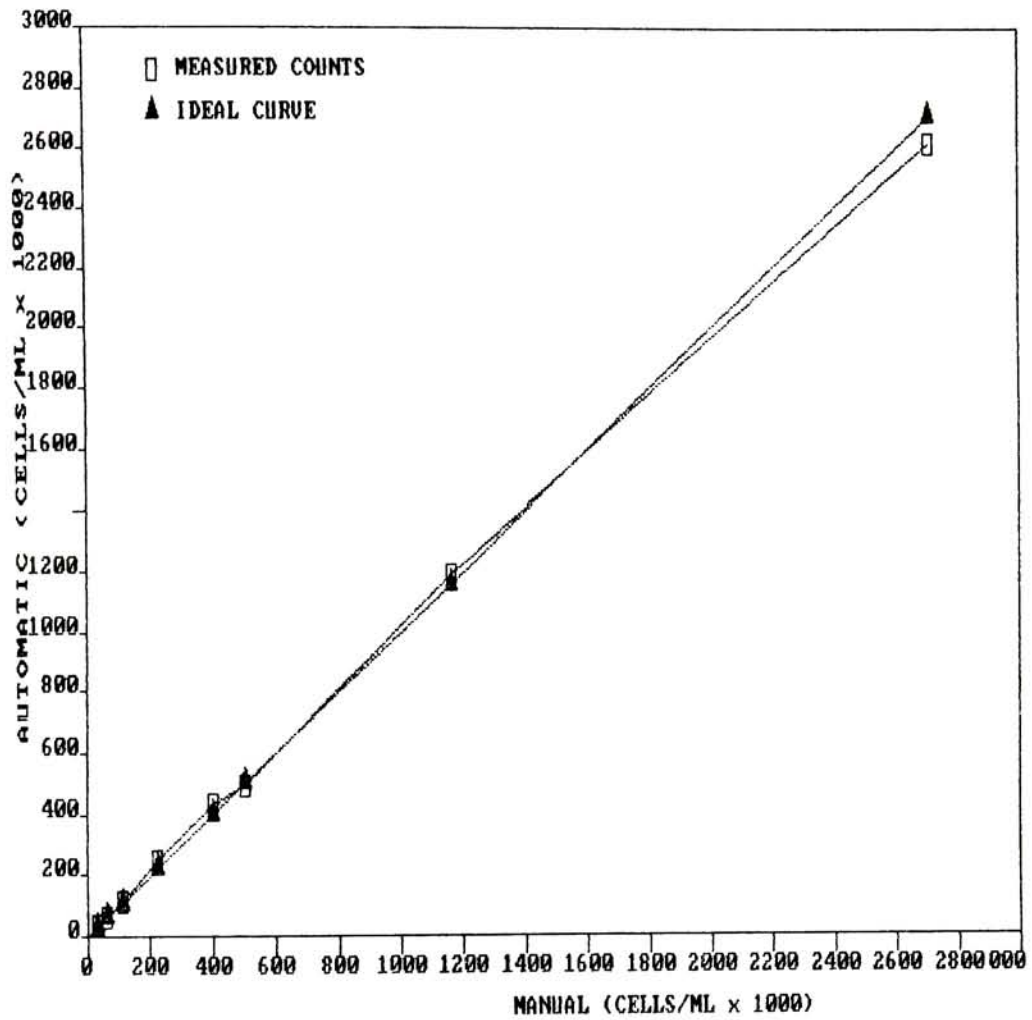
1. Cells of stock solution were counted.
2. Cell solution was diluted over the range of counts seen during the experiment.
3. Counts made both on the hemacytometer and the Coulter counter ZF after (1:100 dilution).
4. Results on the Coulter read absolute cell number per sample, 0-5000 counts.
5. Cell counts as cells/ml reported for both methods.
6. Plots made cell count automatic versus cell count manual.

**Results:**

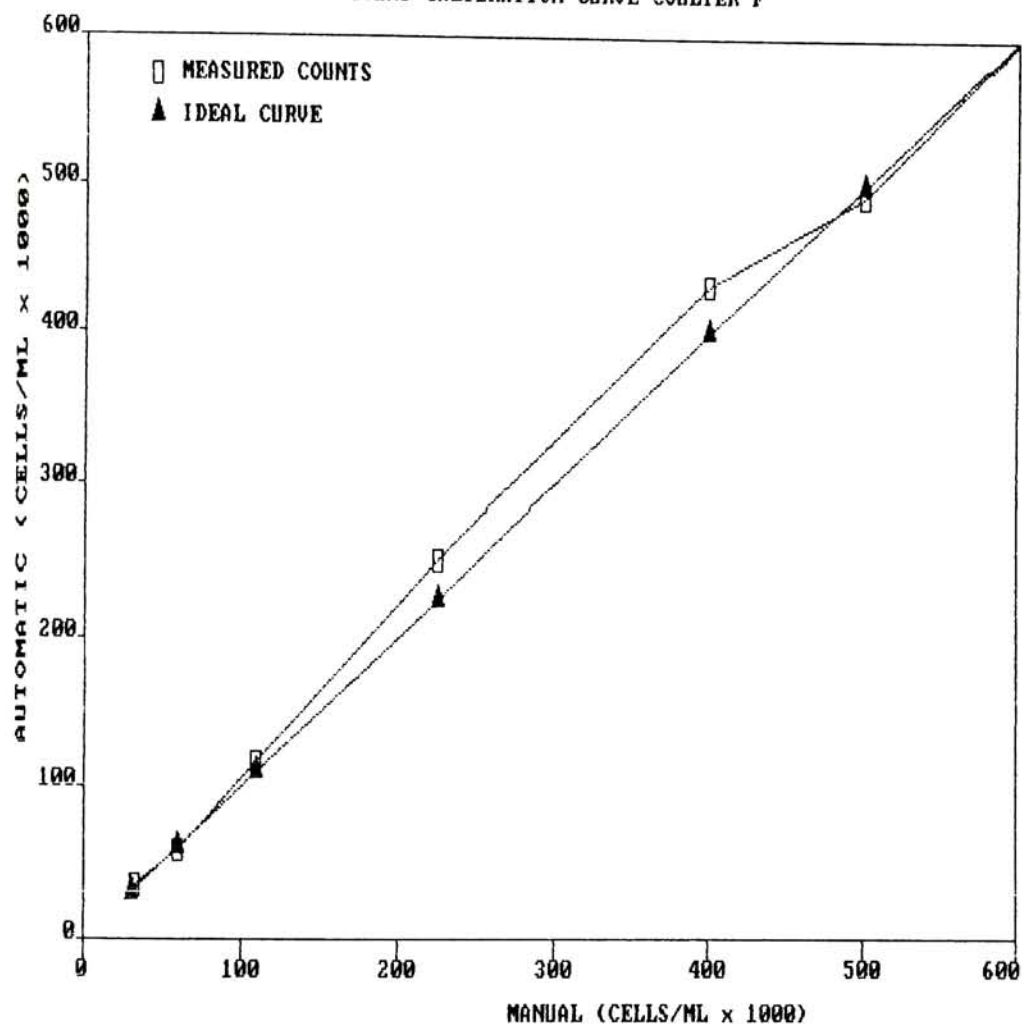
Show counts both methods are the same. See plots 1 and 2.

<u>Counts</u>		<u>Cells/ml</u>
0	=	0
1	=	200
1000	=	200,000
2000	=	400,000
3000	=	600,000
4000	=	800,000
5000	=	1,000,000

CELL COUNT CALIBRATION CURVE COULTER F



CELL COUNT CALIBRATION CURVE COULTER F



**REFERENCES:**

1. Reynolds, C.P., D.C. German, A.G. Wienberg, R.G. Smith. Catecholamine fluorescence and tissue culture morphology: Technics in the diagnosis of neuroblastoma. American Journal of Clinical Pathology 75(3):275-282, 1981.
2. Sumners, C., M.I. Phillips, M.K, Raizada. Rat brain cells in primary culture: Visualization and measurement of catecholamines. Brain Research 264:267-275, 1983.
3. Lindvall, O. and A. Bjorklund. The glyoxylic acid fluorescence histochemical method: a detailed account of the methodology for the visualization of central catecholamine neurons. Histochemistry 39:97-127, 1974.

## APPENDIX 5

### PROTOCOL

#### Preparation of Cells for Catecholamine Analysis

**PURPOSE:** To determine catecholamine levels in C1300 Neuroblastoma (mouse) cells in tissue culture. Particularly, levels of norepinephrine (NE) and dopamine (DA).

#### **MATERIALS:**

##### **Cells:**

NB41A3 C1300 NB cells

##### **Reagents:**

- ice cold phosphate buffered saline, pH 7.4 (Gibco)
- 0.2 M perchloric acid (Mallinckrodt)
- 10 mM (in 0.2 N perchloric acid) dithiothreitol (Sigma)
- 1.0 M potassium hydroxide (to neutralize) (media only)

##### **Equipment:**

Test tubes  
Pipets - serological 5 ml  
Rubber policeman on a glass rod  
Repipets 0-50 ml  
                  100-1000 ul  
Pipet tips  
Centrifuge 8000 g 4°C  
Freeze dryer  
Homogenizer

#### **METHODS:**

1. After removal of growth medium cells are washed 1x with ice cold PBS, pH 7.4.
2. Cells are scraped from tissue culture flask using a rubber policeman.
3. Cells are then transferred to test tubes.
4. An aliquot is removed for cell count and volume in tube is noted and recorded.
5. Cells in test tubes centrifuged supernatant discarded.



## APPENDIX 4

### Fluorescent Catecholamine Staining

**PURPOSE:** To identify and document, visually and photographically, catecholamine (blue green) fluorescence and cellular morphology in C-1300 neuroblastoma cells in tissue culture.

#### **MATERIALS:**

##### **Equipment:**

- American optical fluorescent microscope BG12 exciter filter 405/20 nm band pass filter RKG 455 beam splitting mirror. 460 nm long pass suppression filter.
- Slides and cover slips
- Oven - 85°C
- Warm air source

##### **Reagents:**

- Glyoxylic acid - 50% in H<sub>2</sub>O (#8497, Kodak), 500 gms
- Sucrose (S-5, Fisher Scientific)
- Anhydrous potassium phosphate dibasic (#7092, Mallinckrodt)
- 2M Sodium Hydroxide
- Phosphate buffered saline - pH 7.4 (Gibco) [PBS]
- Triple distilled water

##### **Cells:**

- Mouse C-1300 NB ATCC 147 NB41A3

**Methods** (Combined techniques of Reynolds and Sumners):

1. 4 and 7 day old cultured cells are grown on rectangular glass cover slips.

#### A4.2

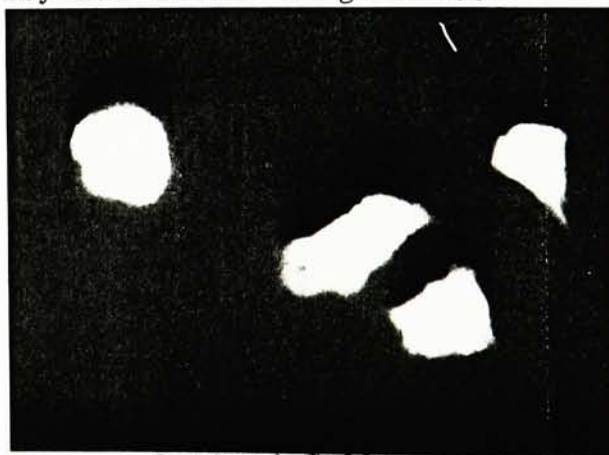
2. The cover slips with cells are removed from the tissue culture flasks and rinsed with PBS, pH 7.4 and
3. immediately placed in a 2% buffered glyoxylic acid solution (SPG) ( $4^{\circ}\text{C}$ ) for 5 minutes.
4. Excess glyoxylic is removed and cover slips dried with compressed air for 10 minutes.
5. Cover slips are heated in oven  $80^{\circ}\text{C}$  for 10 minutes.
6. Cover slips are cooled and mounted on glass slides with liquid parafin.
7. Slides are viewed on A.O. fluorescence microscope - A.O. setting:
  - Exciter filter BG12
  - Barrier filter GG 475
  - Dichroic supp. filter 450 nm
8. Control slides, cells immersed in cold PBS treated and mounted to look at background fluorescence.
9. Resulting all-catecholamine fluorescence and phase contrast cell morphology documented using either instant or 35 mm photography.

#### RESULTS:

1. If blue green fluorescence is present, where, in neurites.
2. Is the mount of fluorescence different in the 4 day and 7 day cells (neurites) - intensity and number of granules.



Unstained Cells



Stained Cells

### A-4.3

#### Positive Control of CA Fluorescence

1. Preparation of SPG solution: Sucrose (5.1 g), anhydrous  $\text{KH}_2\text{PO}_4$  (2.4 g), and glyoxylic acid (0.75 g) were dissolved in 40 ml of distilled water. The pH of the solution was adjusted to 7.4 with 2N NaOH, and the volume adjusted to 75 ml with distilled water.
2. Incubating the cultured cell line C-1300 NB in 4 mg/ml of dopamine in Ham's for 5 minutes on slide.
3. The cells were washed once with Ham's and slide was dried quickly and gently with compressed air.
4. Slides were dipped three times (one dip/sec) in SGP solution. The slides were placed at a  $45^\circ$  angle on a paper towel and the excess solution was blown off the slides with compressed air.
5. A drop of mineral oil was placed on the cell, over which a cover slip was mounted.
6. The slides were heated in an oven at  $95^\circ\text{C}$  for exactly 2-1/2 minutes and allowed to cool at room temperature.
7. The slides were examined with fluorescent microscope.

**REFERENCES:**

1. Reynolds, C.P., D.C. German, A.G. Wienberg, R.G. Smith. Catecholamine fluorescence and tissue culture morphology: Techniques in the diagnosis of neuroblastoma. American Journal of Clinical Pathology 75(3):275-282, 1981.
2. Sumners, C., M.I. Phillips, M.K. Raizada. Rat brain cells in primary culture: Visualization and measurement of catecholamines. Brain Research 264:267-275, 1983.
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#### **MATERIALS:**

##### **Cells:**

NB41A3 C1300 NB cells

##### **Reagents:**

- ice cold phosphate buffered saline, pH 7.4 (Gibco)
- 0.2 M perchloric acid (Mallinckrodt)
- 10 mM (in 0.2 N perchloric acid) dithiothreitol (Sigma)
- 1.0 M potassium hydroxide (to neutralize) (media only)

##### **Equipment:**

Test tubes  
Pipets - serological 5 ml  
Rubber policeman on a glass rod  
Repipets 0-50 ml  
                  100-1000 ul  
Pipet tips  
Centrifuge 8000 g 4°C  
Freeze dryer  
Homogenizer

#### **METHODS:**

1. After removal of growth medium cells are washed 1x with ice cold PBS, pH 7.4.
2. Cells are scraped from tissue culture flask using a rubber policeman.
3. Cells are then transferred to test tubes.
4. An aliquot is removed for cell count and volume in tube is noted and recorded.
5. Cells in test tubes centrifuged supernatant discarded.



## A-5.2

6. 300-400 ul of 0.2 N perchloric acid/10 mM dithiothreitol solution is added to test tubes to deproteinize the tissue.
7. Cells are homogenized.
8. 10 ul sample is obtained for protein determination (ck amount chem lab).
9. The remainder solutions of homogenized cells are centrifuged at 8000 g, 4°C for 20 minutes.
10. Individual supernatants are removed, volumes noted, freeze dried and stored at -80°C until assayed for NE or DA.
11. Samples reconstituted with 20 ul distilled H<sub>2</sub>O.

### REFERENCE:

1. Sumners, C., M.I. Phillips, M.K. Raizada. Rat brain cells in primary culture and measurement of catecholamines. Brain Research 264:267-275, 1983.

### ADDENDUM:

#### Solution preparation

0.2N perchloric acid solution HClO<sub>4</sub> M.W. 100.46  
70% perchloric and stock

$$N = \frac{\text{grams solute}}{\text{liter sln} \times \text{eq. wt.}} \quad \text{weight volume \%} = \frac{\text{wt. grams solute} \times 100}{\text{volume sln ml}}$$

How many ml of 70% perchloric acid stock solution do I need to detail in 10 ml to get a 0.2 N sln?

$$70\% \text{ w/v} = ?N$$

$$W/V = \frac{70 \text{ grams}}{100 \text{ ml}} \quad \frac{700 \text{ grams}}{1000 \text{ ml} \times 1} = 0.7N$$

# A-5.3

To get 0.2 N 1:3.5 dilution of 0.7 N

or

35 ml = 10 ml HCL04/25 ml TD water = 0.2 N HCL04

Now to get 10 mM dithiothreitol in 0.2 N HCL04

N.W. 154.3 (5 gms)

$$\text{No. mmol} = \frac{\text{grams of species} \times 1000}{\text{gm wt}}$$

$$10 = \frac{\text{gm} \times 1000}{154.3}$$

$$\text{gm} = \frac{10 (154.3)}{1000} = \frac{1543}{1000} = 1.543 \text{ g/L}$$

$$.010 \text{ M} = \text{gms GMW/L}$$

or mg M.W./L

$$1 \text{ mM} = .156 \text{ g/L}$$

$$10 \text{ mM} = 1.56 \text{ g/L}$$

0.2 N HCL04 total volume/batch - 35 ml

$$1.56 \text{ g/L or } 0.156 \text{ g/100 ml or } 0.156 \text{ g/100 ml} = x/35 \text{ ml}$$

$$\frac{0.156 \text{ g}}{100 \text{ ml}} = \frac{x}{35 \text{ ml}}$$

$$100x = 5.46$$

$$x = 0.0546 \text{ gms}$$

$$= 0.055$$

## CAT-A-KIT™ assay system (catecholamines [<sup>3</sup>H] radioenzymatic assay)

code TRK.895

### Description

Amersham's catecholamines [<sup>3</sup>H] radioenzymatic assay system provides a very sensitive, reliable and precise quantitative determination of adrenaline (epinephrine), noradrenaline (norepinephrine) and dopamine in urine, plasma and other biological samples. The system utilizes the enzyme catechol-O-methyltransferase (COMT) to catalyse the transfer of a [<sup>3</sup>H]-methyl group from tritiated S-adenosyl-L-methionine ([<sup>3</sup>H]-SAM) to the hydroxyl group in the 3-position of the catecholamines, adrenaline (epinephrine), noradrenaline (norepinephrine) and dopamine. Separation of the resulting products, [<sup>3</sup>H]metanephrine, [<sup>3</sup>H]normetanephrine and [<sup>3</sup>H]methoxytyramine is achieved by thin-layer chromatography. The band corresponding to [<sup>3</sup>H]methoxytyramine is eluted and counted directly, whereas the bands containing [<sup>3</sup>H]metanephrine and [<sup>3</sup>H]normetanephrine are eluted and converted to [<sup>3</sup>H]vanillin by periodate oxidation prior to counting.

As described, the assay measures all three catecholamines separately. However, the assay system can also be used to measure 'total catecholamines' (adrenaline plus noradrenaline) in biological samples.

Adrenaline (epinephrine) and noradrenaline (norepinephrine) may be measured in the range 2-5pg and dopamine in the range 15-20pg per 50µl sample. Each pack contains sufficient material for 90 assay tubes.

- Precise and accurate
- Small sample size: 10-50µl
- No prior extraction required
- Measures total or individual catecholamines.

**For In Vitro Diagnostic Use.**

**Biomedical research**

**Amersham**

2

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3

### Introduction

The three catecholamines adrenaline, noradrenaline and dopamine have different physiological functions varying with their anatomic location. In the nervous system, noradrenaline and dopamine act as neurotransmitters, whereas adrenaline is the major hormone of the adrenal medulla. Catecholamines are known to serve as reliable markers of sympatho-adrenal activity in man<sup>(1,2)</sup>. Measurement of catecholamines can assist in elucidating the role of the sympatho-adrenal system and help in the diagnosis and management of hypertension, coronary disease and acute myocardial infarction. Catecholamine levels are significantly increased when phaeochromocytomas are present and their measurement in plasma can provide clinically useful data when phaeochromocytoma is suspected<sup>(3)</sup>. The availability of Amersham's catecholamines assay system will facilitate the evaluation of the physiological role of these important biogenic amines.

## Summary of the assay

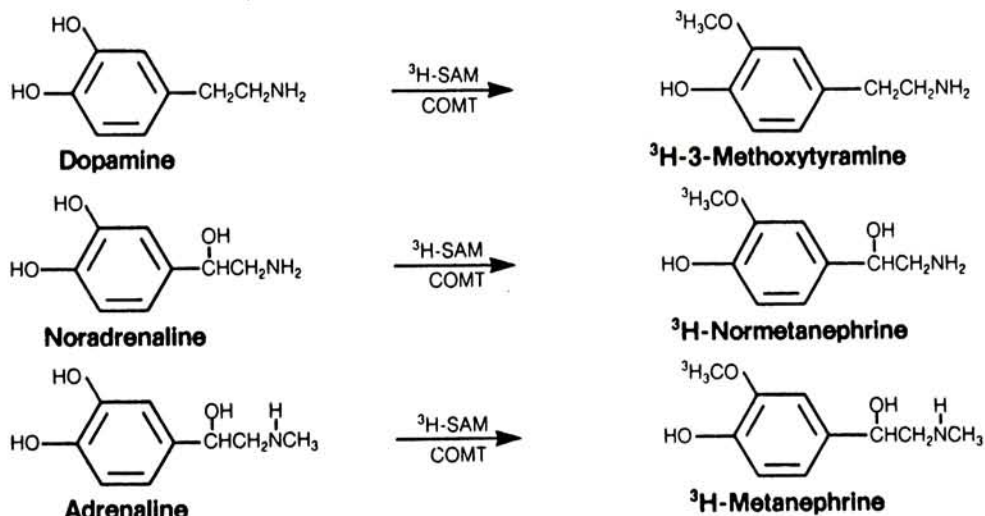
In 1973, Passon and Peuler<sup>(4)</sup> described an innovative single isotope assay for noradrenaline and adrenaline which accurately measured these catecholamines in less than one ml of plasma, required less than two days to complete, and provided sensitivity of less than 200pg of either noradrenaline or adrenaline. Their assay utilized the enzyme catechol-O-methyltransferase (COMT) to catalyze the transfer of a [<sup>3</sup>H]-methyl group from *S*-adenosyl-L-[methyl-<sup>3</sup>H]methionine (<sup>3</sup>H-SAM) to noradrenaline and adrenaline. The resulting products, [<sup>3</sup>H]normetanephrine and [<sup>3</sup>H]metanephrine, respectively, were isolated by thin-layer chromatography. Each labelled derivative was converted by periodate oxidation to [<sup>3</sup>H]vanillin and extracted. The radioactivity attributable to each catecholamine was determined by liquid scintillation counting.

The assay for catecholamines described here follows the basic principles of Passon and Peuler. Extensive modifications of their methodology produced the following improvements:

- 1) The assay will measure dopamine in addition to noradrenaline and adrenaline.
- 2) The sensitivity of the assay is in the range 2-5pg for noradrenaline and adrenaline and 15-20pg for dopamine per 50µl sample.
- 3) Assay time has been significantly reduced.

4) As little as 10-50µl of biological fluid is required. The biochemical principles of the assay are summarized in figure 1.

5

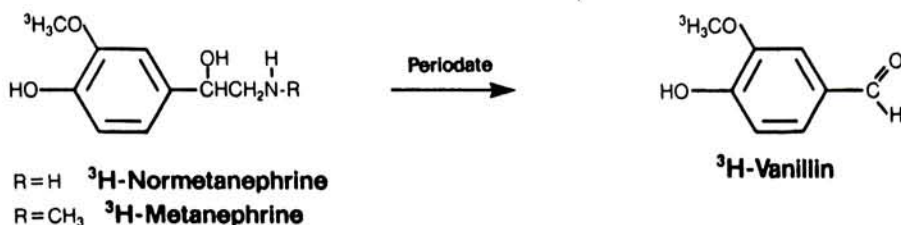


**Figure 1.** COMT–Catalyzed conversion of dopamine, noradrenaline and adrenaline to corresponding [<sup>3</sup>H]methoxy derivatives.

Dopamine, noradrenaline and adrenaline are simultaneously converted to their corresponding meta [<sup>3</sup>H]methoxy derivatives by the catalytic action of a partially purified preparation of COMT in the presence of [<sup>3</sup>H]-SAM.

Following the enzyme-catalyzed O-methylation of the catecholamines, the catecholamine derivatives are extracted, and separated by thin layer chromatography. The isolated catecholamine derivatives, [<sup>3</sup>H]normetanephrine and [<sup>3</sup>H]metanephrine are converted by periodate oxidation to [<sup>3</sup>H]vanillin which is then extracted (figure 2).





**Figure 2.** Periodate oxidation of normetanephrine and metanephrine.

The radioactivity in each extract is proportional to the amount of noradrenaline or adrenaline in the sample being analyzed. Since 3-methoxytyramine is not susceptible to the periodate oxidation, this chemical step serves as the means for differentiating [ $^3\text{H}$ ]3-methoxytyramine (dopamine) from the other two catecholamines.

## Assay methodology

### Contents of the assay system

	90 tube kit
	TRK.895
<b>Vial 1</b> Standard solution	1 Vial
<b>Vial 2</b> Stabilizing solution	1 Vial
<b>Vial 3</b> Buffer solution	1 Vial
<b>Vial 4</b> Tracer ([ $^3\text{H}$ ]-SAM)	2 Vials*
<b>Vial 5</b> Enzyme (COMT)	2 Vials*
<b>Vial 6</b> Stopping solution	1 Vial
<b>Vial 7</b> Sodium metaperiodate	2 Vials*
<b>Vial 8</b> Glycerol	2 Vials*
<b>Vial 9</b> Control plasma (Human)	1 Vial

\* Remove one vial from kit and maintain below  $-20^\circ\text{C}$  in order to avoid unnecessary freezing and thawing.

Amersham's catecholamines assay system provides sufficient reagents for 90 assay tubes. The reagents are stable only when stored below  $-20^\circ\text{C}$  in a non-defrosting freezer. The vials should be removed from the package for thawing and should be refrozen as rapidly as possible. While thawed, vials 1, 4, 5 and 9 should be kept in ice.

The 90 tube kit contains two vials each of [ $^3\text{H}$ ]-SAM (vial 4), COMT (vial 5), sodium metaperiodate solution (vial 7) and glycerol solution (vial 8). Remove one each of vials 4, 5, 7 and 8 from the kit and maintain below  $-20^\circ\text{C}$  in order to avoid unnecessary freezing and thawing.

It is important that cross contamination of reagents not be introduced into any tubes or onto any glassware. Each cap should be replaced on the same reagent vial from which it is removed.

#### Vial 1: standard solution

Vial 1 contains 500 $\mu\text{l}$  of a solution of L-noradrenaline, L-adrenaline and dopamine at 100 $\mu\text{g}$  per ml in acidic glutathione solution. Store below  $-20^\circ\text{C}$ .

The standard solution must be accurately diluted 1:10,000 to a concentration of 10ng/ml for each catecholamine. This solution provides the standard for the assay; 10 $\mu\text{l}$  volumes (100 picograms of each catecholamine) will be used. Due to the instability of the diluted standards, even when stored frozen<sup>(5)</sup>, this diluted solution must be prepared immediately before each use according to the **assay procedure** (step 4).

#### Vial 2: stabilizing solution

Vial 2 contains 500 $\mu\text{l}$  of acidic glutathione solution. Store below  $-20^\circ\text{C}$ .

This solution must be diluted 1:10,000 in the same manner as vial 1 above (**assay procedure**, step 4), and should be made fresh daily. The diluted solution (10 $\mu\text{l}$ ) will be added to every assay sample except those which receive an aliquot of the diluted standard solution.



**Vial 3: buffer solution**

Vial 3 contains 2ml of a ready-to-use solution of Tris buffer pH8.5 containing ethylene glycol-bis ( $\beta$ -aminoethyl ether) N, N'-tetraacetic acid (EGTA) and  $MgCl_2$ . Store below  $-20^\circ C$ .

This solution is used to buffer the enzyme reaction in the assay.

**Vial 4: tracer ( $[^3H]$ SAM)**

Vial 4 contains S-adenosyl-L-[methyl- $^3H$ ]methionine, 9.25MBq, 250 $\mu$ Ci in HCl:ethanol (9:1), pH2.1. Two vials of tracer are provided.

Considerable care should be taken to keep this vial in ice during distribution of this radiochemical to the reagent mixture, after which the vial should be returned promptly to the freezer and stored below  $-20^\circ C$ .

**Vial 5: enzyme (COMT)**

Vial 5 contains 500 $\mu$ l of a ready to use solution of rat liver catechol-O-methyltransferase in Tris buffer containing glutathione, benzylhydroxyamine hydrochloride and dithiothreitol. Two vials of enzyme are supplied. Store below  $-20^\circ C$ .

The COMT enzyme contained in this vial has been isolated from rat liver and partially purified by modification of the method described by Axelrod and Tomchick<sup>(6)</sup>. Enzyme activity is standardized in each COMT preparation by utilizing this assay procedure.

The enzyme concentration is adjusted to provide, in each 10 $\mu$ l aliquot, an excess activity for the purpose of this assay. Slow deterioration of activity may occur due to repetitive thawing and freezing. However, when stored frozen and used according to directions, the excess activity is sufficient to ensure usefulness in this assay until the expiry date.

**Vial 6: stopping solution**

Vial 6 contains 5ml of a ready-to-use solution of normetanephrine, metanephrine and methoxytyramine (each at 4mM) in borate buffer pH11 containing (ethylene dinitrilo) tetraacetic acid disodium (EDTA disodium). Store below  $-20^\circ C$ .

The stopping solution contains each of the products of the enzymatic reaction to serve as carriers for the radiolabelled reaction products. If a precipitate is observed in vial 6, the vial can be gently warmed to dissolve the precipitate.

**Vial 7: sodium metaperiodate**

Vial 7 contains 5ml of a ready-to-use solution of 4% (w/v) sodium metaperiodate. Two vials of this reagent are provided. Store below  $-20^\circ C$ .

**Vial 8: glycerol**

Vial 8 contains 5ml of a ready-to-use solution of 10% (v/v) glycerol. Two vials of this reagent are provided. Store below  $-20^\circ C$ .

In the event that microbiological growth is detected upon thawing, vial 8 should be discarded. A fresh glycerol solution can be prepared (1ml glycerol, diluted to 10ml with glass-distilled, deionized water).

**Vial 9: control plasma (human)**

Vial 9 contains 1ml of control plasma with assayed levels of the three catecholamines (see vial 9 value card for concentrations) plus EGTA and glutathione. Store below  $-20^\circ C$ .

The purpose of this reagent is to permit the user to assess the performance of the kit in his laboratory by obtaining results within the ranges specified on the value card.

The catecholamine levels shown on the value card were determined by replicate assays at time of Quality Control release.

**Note:** If precipitate (fibrin) is observed upon thawing, centrifuge and use supernatant.

**Warnings and precautions**

**For In Vitro Diagnostic Use. Not for Internal or External Use in Humans or Animals.**

**1) Caution: radioactive material**

The radioactive material may be received, acquired, possessed and used only by authorized persons in clinical laboratories or hospitals and only for *in vitro* clinical or laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to humans or animals. Its receipt, acquisition, possession, use, transfer and disposal \* are subject to the regulations and a general licence of Atomic Energy Agencies\*, or the competent official organisation.†

\* In France — L'élimination des déchets radioactifs est réglementée par la législation en vigueur (avis aux utilisateurs paru au Journal Officiel du 6 juin 1970). Le SCPRI ou les organismes désignés par lui, est seul habilité à procéder à la prise en charge des déchets radioactifs. [French]

\* In USA — US Nuclear Regulatory Commission or a State with which the Commission has entered into an agreement for the exercise of regulatory authority. [English]

\* In Canada — Canadian Atomic Energy Control Board. [English]

\* In Canada — La commission de contrôle de l'énergie atomique du Canada. [French]

† In France—Ministère chargé de la Santé, CIREA, SCPRI. [French]

\* In Germany—Für die Bundesrepublik Deutschland: Verordnung über den Schutz vor ionisierenden Strahlen (Strahlenschutzverordnung) vom 13.10.1976. Bundesgesetzblatt 1, Nr. 125 (1976). Unfallverhütungsvorschrift für den Gesundheitsdienst (VBG 103) vom 1.4.1983. [German]

#### **Instructions relating to the handling, use and storage of radioactive materials**

- i) All operations should be carried out in restricted areas by trained and authorized persons. Storage of radioactive materials should be in specially designated areas accessible only to authorized personnel.
- ii) No smoking, eating or drinking should be allowed in the storage or laboratory areas. Pipetting of reagents must not be done by mouth.
- iii) Care should be taken to prevent ingestion or contact with skin and clothing. Use of gloves and protective clothing should be in compliance with local regulatory and laboratory procedures. Wash hands thoroughly after manipulation of test materials.
- iv) Care should be observed to avoid contamination of working areas. If contamination occurs affected areas should be thoroughly washed with an appropriate cleaning agent, for example detergent. Contaminated materials must be disposed of as radioactive waste.

#### **2) Warning—Potentially Infectious Material**

Human blood products provided as components of this pack have been obtained from human donors who were tested individually and who were found to be negative for the presence of Human Immunodeficiency Virus (HIV/HTLV-III/LAV) antibody (Ab) as well as for Hepatitis B surface Antigen (HBsAg) using reliable methods.

As no test method can offer complete assurance that Hepatitis B virus, Human Immunodeficiency Virus (HIV/HTLV-III/LAV) or other infectious agents are absent, all human blood products should be considered potentially infectious. Handling should be in accordance with the procedures defined by an appropriate National biohazard safety guideline or regulation, where it exists (for example USA Center for Disease Control/National Institute of Health manual 'Biosafety in Microbiological and Biomedical Laboratories', 1984).

### **Specimen collection and preservation**

#### **Note: sample medium**

In order to provide the appropriate assay blanks, the sample medium (solution in which the sample is collected, preserved, etc) must be brought to a comparable weight or volume dilution as in the samples to be run. For example, if plasma collected in the special evacuated tube is being run, glass-distilled deionized water (the volume of blood it will draw) should be added to one of these evacuated tubes to provide a comparable concentration for the blank. The control plasma (vial 9) provided in the kit for reference has been prepared with the same sample medium as contained in the special evacuated tubes. Therefore, a blank as prepared above would serve as background for the control plasma. If samples are run together in the assay that have different sample media, blanks must be run for each medium. If further dilution of a prepared sample is required, an equivalent dilution of the sample medium must be provided to be used as the blank. Store prepared sample medium in refrigerator at 4° – 10°C prior to use.

#### **Blood**

Blood may be collected using special evacuated tubes which can be obtained from Amersham (code RPN.532). Alternatively, blood may be collected with a syringe and transferred to a suitable tube, or the user may prefer to use an evacuated tube of his own preparation.

**Warning: It is essential to follow the directions given here exactly, otherwise specimen collection may adversely affect assay results.**

The evacuated tubes available from Amersham contain an anticoagulant, EGTA, and an antioxidant, glutathione. These substances are added to the tubes in a solution (pH 6.0 – 7.4) which contains 90mg/ml EGTA and 60mg/ml glutathione. Twenty microlitres of this solution is needed for each ml of blood to be collected. A standard evacuated tube containing heparin can be used if the proper amount of glutathione is used as stated above. However, other anticoagulants (EDTA, citrate, oxalate) or antioxidants (sodium metabisulphite, ascorbic acid, etc) are not recommended.

#### **Instruction for drawing blood and preparation of plasma**

The hazards of viral hepatitis in laboratories have been recently reviewed<sup>(7)</sup>. Operators should consult this review or other sources for more specific details of proper methods of handling and disposing of specimens, and personal safety.

For convenience a step by step collection and preparation procedure follows:

- 1) After the patient has remained in the supine position in nonstimulating surroundings for at least 30 minutes, draw blood. Take care to avoid haemolysis.
- 2) Invert the tube several times to mix blood with preservatives. **Do not shake.**



3) Immediately place the tube in an ice bath.

4) Centrifuge in a refrigerated centrifuge as soon as possible. (If a refrigerated centrifuge is not available, centrifuge cups or carriers should be pre-chilled).

5) Immediately separate the plasma using a disposable pipette. Take care to avoid the transfer of any cells. Place the clear plasma in a suitable container, and freeze in an upright position. (If the assay is to be completed the same day the specimen may be stored in a refrigerator). Frozen plasma is stable for at least three months when stored in a tightly closed container at a temperature below  $-20^{\circ}\text{C}$ .

**Caution:** In some patients, the venipuncture event may cause plasma catecholamines to rise temporarily, even though the patients are not noticeably nervous or upset. When such responses to venipuncture are anticipated, we recommend the use of an intracath or large bore intermittent infusion set which is placed in the cubital vein or other convenient vein, flushed with a heparin solution (10U/ml), and occluded (heparin lock). When the line for blood drawing is secured, the patient is left recumbent in nonstimulating surroundings for 30 minutes.

At the end of this period, the heparin is flushed from the infusion set by withdrawing a small amount of blood (0.5ml) with a syringe and discarding it. Then, blood is withdrawn. With the needle removed from the syringe, the blood is immediately transferred to a tube containing the additives as described above.

Alternatively, the blood could be withdrawn from the infusion set through a needle holder directly into an evacuated tube.

### Cerebrospinal fluid

Cerebrospinal fluid (CSF) may be added directly in the incubation mixture for the catecholamine analysis. However, it is recommended that immediately upon collection, 20 $\mu\text{l}$  of the EGTA/reduced glutathione solution described in the section on blood collection should be added to each ml of CSF collected. Samples should be stored frozen in a tightly closed container at a temperature below  $-20^{\circ}\text{C}$ .

### Urine

Twelve hour urine samples should be collected in a 2 litre plastic bottle with screw cap (or other suitable bottle) containing 5ml of 6M hydrochloric acid (HCl). The total volume should be accurately measured. If a random urine is collected, the pH should be immediately lowered to between 1 and 2 with concentrated HCl. Remove a suitable aliquot (5ml) and add 20 $\mu\text{l}$  of the EGTA/reduced glutathione solution described previously per ml of urine. Store the urine aliquot containing glutathione below  $-20^{\circ}\text{C}$  until assayed. Due to the sensitivity of the assay, most urine specimens will require accurate dilution prior to use. The actual dilution used will depend on the particular specimen; typical dilutions may be in the range of 1:100. An allowance for the dilution of the urine (including the volume of the added HCl) should be

made when calculating the final concentrations of catecholamines.

### Tissues

Tissue should be frozen in liquid nitrogen or on dry ice immediately upon excision. The tissue should be homogenized in freshly prepared appropriate acid (for example 10ml of cold 0.4N perchloric acid/gram wet weight of tissue) containing 5mM reduced glutathione and then centrifuged in a refrigerated centrifuge at  $0^{\circ}\text{C}$  to produce a 'protein-free' supernatant. This supernatant should be removed and stored frozen in a tightly closed vial or tube. The application of other perchloric acid concentrations or other acids or agents for 'deproteinization' may also be successful. However, it is imperative that a 'tissue-free' sample, which will serve as a blank, be treated under the same conditions, that is, identical volumes of buffer or acid, centrifugation, dilution volumes, etc. as the tissue. Due to the high catecholamine concentrations in many tissues and to the sensitivity of this assay, it will be necessary, in most instances, to dilute the protein-free supernatant with glass distilled deionized water. Typical dilutions for brain tissue may be in the range of 1:20, heart tissue 1:50 and tumour 1:200 or higher.

**Caution:** When adding acidic 'protein-free' supernatant of tissue homogenate to an incubation mixture, be certain that the acidity of that addition will not exceed the buffer capacity of the mixture and denature the enzyme.

## Equipment and supplies required

In addition to the reagents supplied with this kit, the following equipment and supplies are required. The listing of specific products in the following list is not an endorsement of that product, but merely lists products which are available commercially and were used during the development of this assay system.

### Materials and equipment

The following materials and equipment are required:

Test tubes—13 x 100mm and 16 x 125mm (total catecholamines assay only) glass disposable tubes.

Test tube racks for above tubes.

Stoppers, Neoprene, Size 0 to 1.

Pipettes—disposable tip pipettes capable of aliquotting 10, 40 and 50 $\mu\text{l}$ .

Incubation or constant temperature water bath with shaking action capable of holding water temperature at  $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ .

Centrifuge—clinical type ambient temperature to be used with above test tube sizes capable of 1000xg with swinging bucket head.

Vortex mixer.

Thin layer plates—Silica Gel GF, 250 micron thickness; 10 x 20cm plates prescored to eight 2.5 x 10cm plates (Analtech Uniplat, Analtech, 75 Blue Hen Drive, PO Box 7558, Newark, DE19711).

Standard laboratory glassware.

Container for dry ice-alcohol.

Dry ice.

Spotting pipettes or capillaries for manual application of extracted catecholamine derivatives onto thin layer plates.

The application of the extracted catecholamines onto thin layer plates can be facilitated with the use of an automatic spotting device (TLC Multispotter™, Analytical Instrumentation Specialties Supplier, AIS, PO Box 596, Libertyville, IL 60048) with 16 syringe cradle and blower/baffle attachment. Syringes that should be used with the spotter are AIS 250 microliter. Specify 'To be used with Multispotter, CAT-A-KIT™ assay system'.

Developing chamber for development of thin layer plates.

An ultraviolet light source, 254nm wavelength.

Scintillation vials.

Adjustable constant volume dispenser for solvents and solutions.

Shaker evaporator (total catecholamines assay only).

β-Scintillation counter.

Scintillant—a concentrated toluene solution of PPO and POPOP for example, Spectrafluor, available from Amersham Corporation, code 190650 for 500ml and code 190651 for 1 litre, and Liquifluor, New England Nuclear.

#### Solvents and solutions

Toluene analytical reagent (A.R.) grade.

Isoamyl alcohol, A.R. grade.

Spectrafluor, Amersham Corporation, Arlington Heights, IL. (see scintillant, materials and equipment)

Benzene, A.R. grade\*.

Tertiary amyl alcohol, A.R. grade.

Methylamine solution (40% in water).

0.1M acetic acid.

Ethyl alcohol, absolute.

0.05M Ammonium hydroxide (make fresh regularly).

Glass-distilled, deionized water.

#### Solvent mixtures to be prepared

1) Toluene/isoamyl alcohol (3:2) (v/v).

2) Tertiary amyl alcohol/benzene\*/methylamine solution (6:2:3) (v/v/v).

3) Toluene/Spectrafluor (1000:50) (v/v).

4) Toluene/isoamyl alcohol/Spectrafluor (700:300:50) (v/v/v).

\*If benzene is not available, a solvent system consisting of chloroform/methanol/ethylamine (32:6:4, v/v/v) may be substituted.

## Assay procedure

### Differential catecholamines assay

1) Thaw and examine samples. If they are not clear, centrifuge to free the sample of particulate matter.

**Caution: Presence of haemolysis, cloudiness or solid matter in specimens may give invalid results.**

Dilute those samples which require dilution with distilled water. **All distilled water used here and in subsequent steps of the assay must be glass distilled deionized water.** If sample dilution is required, an equivalent dilution of the sample medium must be assayed as the blank.

2) Determine the number of tubes (13 x 100mm) required from table 1. Number each tube and place in an ice water bath.

3) Aliquot two (2) 50µl portions of each sample (B in table 1) into the appropriate tubes (when performed in duplicate a total of 4 tubes per sample will result). Include vial 9 as a sample to assess the performance of the kit. Where samples are run using a different media, separate blanks must be run for each medium.

4) Dilute a portion of vial 2 1:10,000 with distilled water. Add 10µl to the first aliquot of each sample (C in table 1). Similarly dilute a portion of the vial 1 1:10,000. Store in ice bath. Add 10µl of the diluted standard solution to the second aliquot of each sample (D in table 1).

5) Prepare a reagent mixture in a suitable disposable tube with the following components added in the following order and proportions:

Distilled water	10µl
Vial 3	10µl
Vial 4	10µl
Vial 5	10µl
For each tube	40µl

Multiply the volume of each reagent by the number of tubes determined in step 2 plus a two tube excess for every run. Vortex the mixture lightly before adding vial 5, COMT, and lightly again when the reagent mixture is complete. Add 40µl of the reagent mixture (E in table 1) to all tubes last.

6) Mix lightly. Centrifuge briefly (300 x g for 30 seconds). Incubate all tubes at 37°C for 1 hour in a shaking water bath.

7) After incubation, return the tubes to the ice bath. Add 50µl of vial 6 to each tube and mix vigorously. (The stopping solution consists of each of the products of the enzymatic reaction normetanephrine, metanephrine and methoxytyramine at a concentration of 4mM, to serve as carriers for the radiolabelled reaction products).

Number a second set of 13 x 100mm test tubes. (16 x 125mm tubes required if only total catecholamines are to be determined).



**8)** Add 100µl of 0.1M acetic acid to each of the second set of numbered test tubes.

**9)** Add 2ml of toluene/isoamyl alcohol (3:2 v/v) to the incubation tubes, mix vigorously and centrifuge (2 minutes at 800 xg; subsequent centrifugations should be performed in the same manner). Two distinct layers should result. Quick freeze (15 seconds) each tube in a dry ice-alcohol\* bath.

**10)** Remove tube from the bath, blot outside of tube to prevent dripping of alcohol, and decant the upper organic phase into the corresponding tube containing the acetic acid solution prepared in step 8. Discard lower aqueous phase.

**11)** Mix vigorously, centrifuge and freeze as in step 9. Aspirate off the upper organic phase and discard.

\*Ethanol, methanol or acetone may be used in the dry ice-alcohol bath.

**12)** Thaw the aqueous acetic acid phase, add 1ml toluene/isoamyl alcohol (3:2), mix vigorously, centrifuge as above, quick freeze, aspirate and discard the upper organic phase.

**For total catecholamines assay go to page 20, for completion of differential determination—proceed to step 13.**

**13)** Add 100-150µl of absolute ethanol to the thawed acetic acid from step 12 to facilitate evaporation. Mix until a clear solution results. Centrifuge at 800 xg for 1 minute.

**14)** Prior to spotting, scribe channels on the thin layer plate for desired number of samples. Number each lane omitting outer lanes (see figure 3). Apply all of the acetic acid/ethanol solution from step 13 1.5-2.0cm from the bottom of the scribed thin layer plate: (prescored plates need not be separated until step 17).

**Table 1. Quantity of each reagent required per tube**

	Number of tubes	A Sample medium	B Sample	C Diluted stabilizing solution	D Diluted standard solution	E Reagent mixture
Blank	2	50µl	—	10µl	—	40µl
Sample(s)	2	—	50µl	10µl	—	40µl
Sample(s) + standard	2	—	50µl	—	10µl	40µl

The acetic acid/ethanol solution should preferably be applied as a single spot using an automatic TLC spotter. Alternatively, it may be applied manually with a capillary, syringe or spotting pipette; in this case, the sample should be streaked on the plate. Keep the band or spot as narrow or small as possible to achieve the best resolution. A warm air stream is recommended.

**15)** Place the plate(s) in a paper-lined TLC developing tank. Make certain that the solvent is at least 1cm below the spots. Develop the TLC plates with a solvent consisting of tertiary amyl alcohol/benzene\*\*/methylamine solution (6:2:3, v/v/v). Prepare solvent immediately prior to use to obtain best separation. Permit the solvent to migrate to the top of the plate. More than one plate may be developed in the same tank at one time.

**16)** Remove the plate(s) from the tank and dry thoroughly; discard the developing solvent. Visualize the zones under the 254nm ultraviolet light. The top zone contains 3-methoxytyramine; the middle zone contains metanephrine; the bottom zone contains normetanephrine (see figure 3). Outline the zones in the silica gel with the tip of a sharp object without disturbing the zones.

**17)** Assemble in appropriate quantity three sets of scintillation vials; one each for the adrenaline, noradrenaline and dopamine assay. Carefully separate the sections of the TLC plate at the prescored lines and carefully scrape off the silica gel from each zone into a

correspondingly numbered scintillation vial. Each set of the scintillation vials should now contain the silica scraping for set 1) 3-methoxytyramine; set 2) metanephrine, and set 3) normetanephrine, respectively.

From this point on the assay for dopamine differs from that of adrenaline and noradrenaline. Since the procedure for the assay of adrenaline and noradrenaline from the silica gel scrapings containing the [<sup>3</sup>H] metanephrine and [<sup>3</sup>H] normetanephrine utilizes the same steps and conditions, the assay of these two sets of samples may be carried out simultaneously. A separate section describing each assay is detailed to prevent any confusion in the processing of the isolated labelled derivatives.

#### **Dopamine assay**

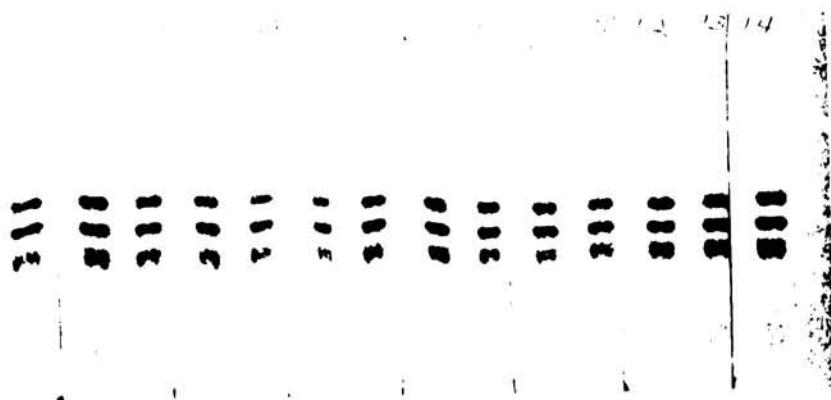
**18)** To the scintillation vials containing silica gel from the top zone, add 1ml of 0.05M ammonium hydroxide to each vial to elute the methoxytyramine from the silica gel. Mix vigorously.

**19)** Add 10ml of toluene/isoamyl alcohol/Spectrafluor (700:300:50, v/v/v) to each vial, cap the vials, and shake vigorously.

**20)** Dark control the vials and count in a liquid scintillation counter. These vials contain radioactivity from dopamine.

\*\*If benzene is not available a solvent system consisting of chloroform/methanol/ethylamine (32:6:4, v/v/v) may be substituted.





**Figure 3.** Application of [ $^3\text{H}$ ]-methoxycatecholamines to silica gel thin layer and chromatographic separation. Thin layer plate (20 x 10cm) illustrating separation of [ $^3\text{H}$ ]-methoxycatecholamines from 14 samples which were spotted simultaneously by TLC Multispotter. Note that the outside lane on each 20 x 10cm plate (prescored to eight 2.5 x 10cm lanes) is not used due to frequent variations in uniformity of silica gel layer in these edges. All plates were visualized under ultraviolet light.

#### Adrenaline assay

**21)** To the scintillation vials containing the silica gel from the middle zone (metanephrine) add 1ml of 0.05M ammonium hydroxide to each vial to elute the amine from the silica gel. Mix vigorously (vortex or equivalent).

**22)** To initiate the periodate oxidation step add 50 $\mu\text{l}$  of vial 7 to each vial at timed intervals and mix vigorously. Periodically shake the vials during the reaction.

**23)** Five minutes after the addition of periodate to the first vial in step 22 above, add 50 $\mu\text{l}$  of vial 8 to each vial maintaining the order and time interval established in step 22.

**24)** Add 1ml of 0.1M acetic acid to each vial and mix. With adequate ventilation, add 10ml of toluene/Spectrafluor (1000:50 v/v) to each scintillation vial.

**25)** Cap the vials and shake vigorously. Dark control the vials and count in a liquid scintillation counter. These vials contain the radioactivity from adrenaline.

#### Noradrenaline assay

**26)** To the scintillation vials containing the silica gel from the bottom zone (normetanephrine) add 1ml of 0.05M ammonium hydroxide to elute the amine from the silica gel. Mix vigorously (vortex or equivalent).

**27)** To initiate the periodate oxidation step add 50 $\mu\text{l}$  of vial 7 to each vial at timed intervals and mix vigorously. Periodically shake vials during the reaction.

**28)** Five minutes after the addition of periodate to the first vial in step 27 above, add 50 $\mu\text{l}$  of vial 8 to each vial maintaining the order and time interval established in step 27.

**29)** Add 1ml of 0.1M acetic acid to each vial and mix. With adequate ventilation, add 10ml of toluene/Spectrafluor (1000:50 v/v) to each scintillation vial.

**30)** Cap the vials and shake vigorously. Dark control the vials and count in a liquid scintillation counter. These vials contain the radioactivity from noradrenaline.

### Total catecholamines assay

If the differential determination of the three catecholamines is not desired, total catecholamines (noradrenaline and adrenaline) can be determined.

**31)** For the total catecholamines assay, the procedure is identical to that used for the differential assay (steps 1 through 12). The remainder of the procedure follows here.

**32)** Dry the acetic acid solution from step 12 under a stream of air or under reduced pressure.

**33)** Add 1ml of 0.05M ammonium hydroxide to the dried residue (which may not be visible) and vortex vigorously.

**34)** To initiate the periodate oxidation step add 50µl of vial 7 to each tube at timed intervals and mix vigorously. Periodically shake tube rack during reaction.

**35)** Five minutes after the addition of the periodate to the first tube in step 34 above, add 50µl of vial 8 to each tube.

**36)** Add 1ml 0.1M acetic acid to each tube and mix. With adequate ventilation, add 10ml of toluene/Spectrafluor (1000:50 v/v) to each tube, stopper and shake vigorously for 30 seconds. Centrifuge for 2 minutes at 800xg and freeze in a dry ice alcohol bath for approximately 1 minute.

**37)** Decant the upper organic phase from each tube into a separate scintillation vial containing 2ml of 0.1M acetic acid. Cap the vials and shake each vigorously. Dark control the vials and count in a liquid scintillation counter. Discard the lower (aqueous) phase remaining in the test tubes.

**38)** The vials at this point contain the radioactivity from noradrenaline plus adrenaline (since dopamine is not susceptible to the periodate oxidation, it does not contribute to the radioactivity in these samples. Its presence in the standard solution does not affect the results). Please note section on typical data and calculations for the total catecholamines assay. The picograms of standard in the calculation equations is the sum of noradrenaline plus adrenaline.

### Procedural notes

#### Order of addition of reagents

The order of addition indicated in steps 3 through 5 is recommended. Particular care should be taken to vortex the reagent mixture after the addition of vial 4, (step 5) which is strongly acid, so as not to layer the enzyme onto the acid and cause extensive denaturation.

#### Standard

The catecholamines assay has been shown to be linear to 3000pg per assay sample for noradrenaline (NA), adrenaline (A), and dopamine (DA), when these standards were added to samples from a plasma pool at 0.5 log intervals.

#### Dilution of standards and samples

The dilution specified in step 4 produces a standard with a content of 100pg of each catecholamine in the 10µl aliquot added to the incubation mixture. Although it is recommended that 100pg be used as the standard, the concentration of the standard may be adjusted to be compatible with the quantity of catecholamines anticipated in the sample.

Further dilution of the sample with diluted vial 2 or distilled water would be more appropriate than increasing the standards. If any sample appears to contain more than 3000pg of any catecholamine it should be diluted appropriately with the diluted vial 2

(step 4) and repeated. In the event that the catecholamine concentration in the sample is quite high, the relative contribution of the standard to the total counts may be greatly decreased from that in other samples or may not even be detectable.

#### Mixing

Since this assay is dependent at several steps upon partitioning of labelled product from one phase to another, the action should be severe enough to ensure thorough mixing of the phases. The best mixing is achieved by several brief vortexings with a pause between them. Ideally total time is approximately 15 seconds.

#### Recommended stopping points

Sometimes the operator may not be able to complete the differential assay in a single working session. Steps in the procedure where the assay may be terminated for the session are **a)** following step 12, at which time the catecholamines have been extracted and are in acetic acid (stopper tubes and store below -20°C), and **b)** after step 16 or 17, the TLC separation of catecholamines. If the interval at **b)** will be longer than overnight, it is recommended that the TLC plates be placed in a desiccator and frozen.

#### Radioactivity counting

It is not necessary to separately count instrument background. The background is part of the assay blank and is therefore subtracted in the calculations.

The user should count each vial for a period of time sufficient to achieve good counting statistics. Amersham recommend a counting time of 10 minutes. Under exceptional circumstances a counting time of 4 minutes will provide acceptable results. In general, Amersham urges users to count as long as possible to reduce this source of error in their final results.

#### Inhibition of O-methylation

Some plasma samples may appear to inhibit the O-methylation of the catecholamines as evidenced by a low increment of radioactivity attributable to the added standard (CPM/picogram standard). If an error in delivering the standard can be ruled out, that sample should be diluted (that is 1:3 or 1:10) with distilled water.

#### Limitations of use

Catecholamine levels can be falsely elevated if the samples are not collected as instructed (see page 10). Please take particular care when collecting these samples to allow an accurate representation of catecholamine levels (see pages 11, 12 and 15).

Some medications have a direct or indirect interference on the catecholamine assay. Please refer to appendices I and II for information.

Tissue measurements of catecholamines require extreme care as it pertains to batch sample extraction and preparation of this extraction for radioenzymatic assay. Please note the section discussing tissue preparation on page 13.

### Typical data and calculations

#### A. Differential determination

The data summarized in table II provides an example for the calculation of catecholamines content in plasma.

Table II. Typical differential assay data

Sample no.	Description	Radioactivity (CPM)		
		NA	A	DA
1	Blank	31	28	184
2	Blank	36	28	181
3	50µl Plasma #1	402	238	275
4	50µl Plasma #1	449	254	329
5	50µl Plasma #1 + Std	3259	3397	2964
6	50µl Plasma #1 + Std	2617	3058	2852

All standards are 100 picograms

#### Calculations

Average data description	Radioactivity (CPM)		
	NA	A	DA
Blank	33	28	183
Plasma #1	425	246	302
Plasma #1 + Std	2938	3228	2908

Catecholamine concentration (pg/ml) =

$$\frac{\text{CPM sample} - \text{CPM blank}}{\text{CPM (sample + std)} - \text{CPM sample}} \times \frac{\text{pg standard}}{\text{ml sample vol.}}^{**}$$

#### Plasma sample 1 (differential)

$$\text{NA } \frac{425-33}{2938-425} \times \frac{100}{0.05} = 312 \text{ picograms/ml}$$

$$\text{A } \frac{246-28}{3228-246} \times \frac{100}{0.05} = 146 \text{ picograms/ml}$$

$$\text{DA } \frac{302-183}{2908-302} \times \frac{100}{0.05} = 92 \text{ picograms/ml}$$

**\*\*Note:** If dilution of sample is required, be sure to correct for dilution factor.



## B. Total catecholamines

The data summarized in table III provides an example for the calculation of total NA and A in the total catecholamines assay.

**Table III. Typical total catecholamines data**

Sample no.	Description	Radioactivity (CPM)
		Total NA + A
1	Blank	106
2	Blank	86
3	50µl Plasma #1	880
4	50µl Plasma #1	907
5	50µl Plasma #1 + Std	7464
6	50µl Plasma #1 + Std	7668

All standards are 200 picograms (sum of concentrations of NA and A).

### Calculations

Average data description	Radioactivity (CPM)
	Total NA + A
Blank	96
Plasma #1	894
Plasma #1 + Std	7566

Catecholamine concentration (pg/ml) =

$$\frac{\text{CPM sample} - \text{CPM blank}}{\text{CPM (sample + std)} - \text{CPM sample}} \times \frac{\text{pg standard}^*}{\text{ml sample vol.}^{**}}$$

### Plasma sample 1 (total catecholamines NA + A)

$$\frac{894 - 96}{7566 - 894} \times \frac{200}{0.05} = 478 \text{ picograms/ml}$$

**\*Note:** In this calculation the Std is sum of NA and A which is 200pg.

## Additional Information

### Performance data

#### Standard

The radioactivity produced by the standards in the results listed (page 23) was obtained utilizing a stock CAT-A-KIT™. Radioactivity in the standards will vary with the specific activity of the substrate [<sup>3</sup>H]-SAM and also because of variations in the O-methylating efficiencies of the three substrates, efficiency of extraction, and variations in scintillation counting efficiency with the various solvents. The extent of the labelling in the three standards will not be equal but they should approximate the proportions or ratios indicated in the typical data.

#### Use of standards

This assay is dependent upon the efficiency of the COMT enzyme to transfer the labelled methyl group from [<sup>3</sup>H]-SAM to the catecholamines. Therefore, it is necessary to add a standard to a second aliquot of the sample to accurately assess the methylating efficiency. EGTA has been added to the incubation mixture to increase the efficiency of the methylation in the presence of plasma.

#### Interfering substances

Although the extraction steps, together with the thin layer chromatographic separation and periodate oxidation, provide specificity for the catecholamines assayed, catechol-containing compounds may interfere. The drugs listed in appendix I and/or their

metabolites may give a positive response in this assay. This may be most noticeable in the total catecholamines assay. Common interfering substances are α-methyldopamine and α-methylnoradrenaline which are metabolites of α-methyldopa\* (Aldomet®, Merck), and isoproterenol. In addition, the drugs listed in appendix II may alter endogenous catecholamine levels.

**Caution:** Radiopaque materials (X-ray contrast media) which contain iodine may interfere with the enzymatic derivatization of the catecholamines. Specimens for catecholamine analysis should not be drawn until such materials have been cleared from the circulation.

#### Crossover of catecholamine assays

The extent of crossover of one catecholamine into the assay of a second has been determined by the addition of a single catecholamine standard (as obtained commercially without further attempts at purification) to a plasma-containing incubate.

Crossover on the basis of total radioactivity produced by the standard is summarized as follows:

\*α-methyldopa itself does not interfere.

Table IV. Assay crossover

Crossover	Mean
DA - NA	<0.1%
A - NA	1.3%
DA - A*	<0.1%
NA - A	0.2%
NA - DA	<0.1%
A - DA	<0.1%

Each mean is the average of 6 determinations.

\*Samples with elevated levels of dopamine could show higher crossover into adrenaline<sup>(8)</sup>.

### Sensitivity

The data summarized in the section on sample calculations provides an indication of the sensitivity of the differential assay. The radioactivity in the respective blanks is equivalent to approximately 1.2pg NA, 0.9pg A and 7.1pg DA. Thus, the realised sensitivity of the assay (at twice the radioactivity equivalents in the blank) is then NA, 2.4pg; A, 1.8pg and DA, 14.2pg per 50µl sample assay. Expected sensitivity will range from 2.5pg for NA and A and 15-20pg for DA per 50µl sample.

### Precision

#### Variation within runs

As measured in groups of four for additions of standards at 0.5 log intervals from a blank (0pg) to 3000pg the average coefficient of variation was NA, 4.2%; A, 3.6% and DA, 4.6%.

#### Variation between runs

As measured 35 times over a period of one month, the coefficient of variation for between assay performance of a human plasma sample spiked with three catecholamines were: NA, 7.5%, A, 10% and DA, 12.6%. The individual values in these experiments were NA, 708pg/ml  $\pm$  53; A, 509pg/ml  $\pm$  47; DA, 444pg/ml  $\pm$  56 (mean  $\pm$  S.D.). A second human plasma spiked with DA only was measured 16 times over 7 weeks resulted in coefficients of variation for NA, 7.8%; A, 13.5% and DA, 11.8%. Individual values for these experiments were NA, 191pg/ml  $\pm$  15; A, 53pg/ml  $\pm$  7 and DA, 228pg/ml  $\pm$  27. Relatively high coefficients of variation in catecholamine levels determined by this methodology may be observed when these levels approach the limit of sensitivity of the assay, for example, DA in the plasma of normal patients has been discussed by Michaels<sup>(9)</sup>.

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### Appendix I

#### Medications with direct interference in radioenzymatic assay

Aerolone\* (cyclopentamine and isoproterenol hydrochloride, Lilly)  
 Aldoclor\* (methyldopa and chlorothiazide, Merck)  
 Aldomet\* (methyldopa, Merck)  
 Aldoril\* (methyldopa and hydrochlorothiazide, Merck)  
 Alupent\* (metaproterenol sulfate, Boehringer Ingelheim)  
 Bendopa™ (levodopa, ICN)  
 Brethine™ (terbutaline sulfate, Geigy)  
 Bronkephrine\* (ethylnorepinephrine hydrochloride, Breon)  
 Bronkometer\* (isoetharine mesylate and phenylephrine, Breon)  
 Bronkosol\* (isoetharine hydrochloride and phenylephrine, Breon)  
 Cobefrin\* (nordephrin hydrochloride, Winthrop)  
 Dopar\* (levodopa, Eaton)  
 Duo-Medihaler\* (isoproterenol hydrochloride and phenylephrine bitartrate, Riker)  
 Intropin\* (dopamine hydrochloride, Arnar-Stone)  
 Isuprel\* (isoproterenol hydrochloride, Winthrop)  
 Larodopa\* (levodopa, Roche)  
 Levophed\* (levarterenol bitartrate, Winthrop)  
 Luf-Iso™ (isoproterenol sulphate, Mallinckrodt)  
 Medihaler-Epi\* (epinephrine bitartrate, Riker)  
 Medihaler-Iso\* (isoproterenol sulphate, Riker)  
 Metaprel\* (metaproterenol sulphate, Dorsey)  
 microNEFRIN\* (racemic epinephrine, Bird)  
 Norisodrine\* (isoproterenol sulphate, Abbott)  
 Proterol\* (isoproterenol hydrochloride, Key)  
 Sinemet\* (carbidopa and levodopa, Merck)  
 Sus-Phrine\* (epinephrine, Cooper)  
 Vaponefrin\* (racemic epinephrine, Fisons)  
 Vap-N-Iso\* (isoproterenol hydrochloride, Fisons)  
 Ventaire\* (protokylol hydrochloride, Marion)

### Appendix II

#### Medications with possible indirect interference in measurement of catecholamines

Amphetamines and other anorexics  
 Antidepressants  
 Antiasthmatics  
 Antianginals  
 Bronchial dilators  
 Phenethylamine and similar compounds  
 Hypotensives  
 Systemic Decongestants  
 Psychostimulants  
 Sympathomimetics  
 Reserpine Alkaloids  
 Vasodilators

#### Possible decrease in catecholamines

Antiarrhythmics  
 Hypotensives  
 Reserpine alkaloids

#### Possible effect on catecholamines, unknown direction of change

Anesthetics  
 Anticonvulsants  
 Antihistamines  
 Diuretics  
 Cough and Cold Formulations  
 Sedatives, barbiturates and others

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# CAT-A-KIT™ assay system (Catecholamines [<sup>3</sup>H] radioenzymatic assay)

code TRK.895

## Contents

Each pack contains the following components:

- 1 Vial (vial 1) containing standard catecholamines; 100µg per ml each of L-noradrenaline, L-adrenaline and dopamine in acidic glutathione solution.
- 1 Vial (vial 2) containing stabilizing solution, acidic glutathione solution
- 1 Vial (vial 3) containing assay buffer, a ready to use solution of Tris buffer pH8.5 containing ethylene glycol- bis (β-aminoethyl ether) N, N'-tetraacetic acid (EGTA) and MgCl<sub>2</sub>
- 2 Vials (vial 4) of S-adenosyl-L-[methyl-<sup>3</sup>H] methionine, 9.25MBq; 250µCi in HCl ethanol 9:1, pH2.1.
- 2 Vials (vial 5) of enzyme; a ready to use solution of rat liver catechol-O-methyltransferase in Tris buffer containing glutathione, benzylhydroxyamine hydrochloride and dithiothreitol.
- 1 Vial (vial 6) of a ready to use solution of stopping solution; 4mM each of normetanephrine, metanephrine and methoxytyramine in borate buffer pH11 containing EDTA.
- 2 Vials (vial 7) of a ready to use solution of 4% w/v sodium metaperiodate
- 2 Vials (vial 8) of a ready to use solution of 10% v/v glycerol.
- 1 Vial (vial 9) of control plasma (human) with added EGTA and glutathione
- Value card.
- Instructions for use

Each pack contains sufficient material for 90 assay tubes.

## Storage

Store the reagents below -20°C in a non-defrosting freezer.

## Availability

From stock.

## Expiry

The expiry date is stated on the package and will normally be at least four weeks from the date of despatch.

## Related products

Amersham supply a number of [<sup>3</sup>H] SAM products:

S-Adenosyl-L-[methyl- <sup>3</sup> H] methionine	TRK 865
S-Adenosyl-L-[methyl- <sup>3</sup> H] methionine	TRK 581
S-Adenosyl-L-[methyl- <sup>3</sup> H] methionine	TRK 614
S-Adenosyl-L-[methyl- <sup>3</sup> H] methionine	TRK 236

as well as [<sup>14</sup>C]-SAM, [<sup>3</sup>H] dopamine and [<sup>3</sup>H] noradrenaline.

S-Adenosyl-L-[2- <sup>14</sup> C] methionine	CFA 697
S-Adenosyl-L-[carboxyl- <sup>14</sup> C] methionine	CFA 477
S-Adenosyl-L-[methyl- <sup>14</sup> C] methionine	CFA 360,
	CFA 428

[2,5,6- <sup>3</sup> H] Dopamine	TRK 284
[7,8- <sup>3</sup> H] Dopamine	TRK 582
DL-[7- <sup>3</sup> H]Noradrenaline	TRK 174
L-[7,8- <sup>3</sup> H]Noradrenaline	TRK 584

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# Coulochem Applications

## Plasma Catecholamines

**the entire procedure from start of extraction to the final result is under 30 minutes.**

Continuously elevated levels of norepinephrine and dopamine are indications of certain neurological disorders and adrenal medulla tumors. It has become prudent to monitor catecholamine levels in certain hypertensive patients since hypertension may result from adrenal medulla tumors. These tumors may be treatable if diagnosed early in their formation.

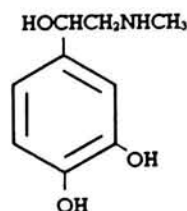
Various technologies have been utilized to measure plasma catecholamine levels. Most have proved to be costly and time-consuming. Recently, liquid chromatography with electrochemical detection has proved to be a cost-effective and reliable means for performing these determinations.

A drawback of early electrochemical determinations of catecholamines was the interference by uric acid. Uric acid co-eluted with norepinephrine in many chromatographic systems, and it was sometimes impossible to separate them. Another problem with detection of the catecholamines was the laborious sample preparation required to isolate and concentrate the catecholamines.

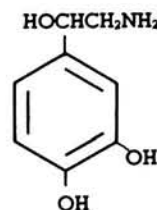
The ESA Coulochem® Multi-Electrode Electrochemical Detector provides a means of electrochemically separating interfering substances, such as uric acid, from the components of interest.

ESA has modified the procedure of Anton and Sayre so that the entire analysis from start of extraction to final result is under 30 minutes.

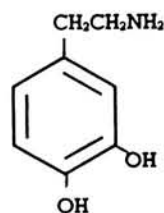
**Figure 1.**  
**Structures of the three catecholamines, epinephrine, norepinephrine, and dopamine.**



**Epinephrine**



**Norepinephrine**

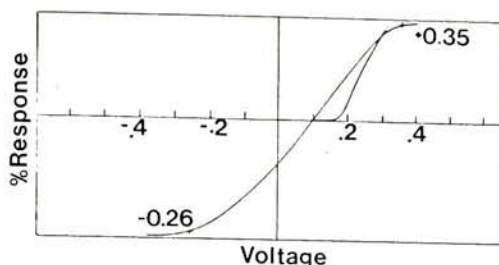


**Dopamine**

Because of the unique coulometric design of the Coulochem, virtually 100% of each compound is oxidized or reduced by each electrode. By selective choice of potentials, one or more compounds may be removed at upstream electrodes, leaving only the specific compounds of interest at the downstream or analytical electrode.

**Figure 2.**

**Current-voltage curve for epinephrine, norepinephrine, and dopamine with the corresponding curve for uric acid.**



The (current-voltage) curve describes the behavior of a compound of interest to oxidation and/or reduction potentials.

The approximate potentials for this analysis were derived from a current-voltage curve determined by experimentation. To produce a C-V curve, a constant mass is injected while the applied potential is varied. The curve (Figure 2) describes the behavior of a compound of interest to oxidation and/or reduction potentials.

The optimum potentials for the three catecholamines of interest are: -0.26 V for reduction and +0.35 V for oxidation. However, to eliminate the effects of uric acid, the following oxidation and reduction potentials were used:

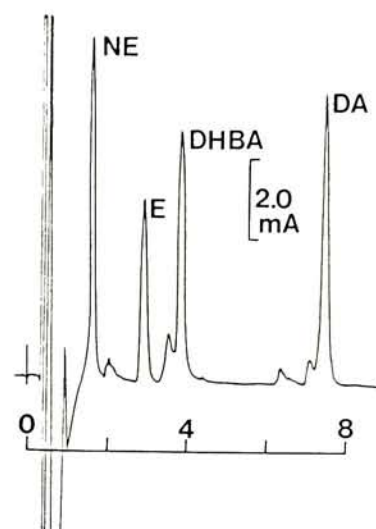
Conditioning Cell = + 0.4 V  
 $T_1$  = + 0.1 V  
 $T_2$  = - 0.26 V

With these potentials, the compounds are fully oxidized at the conditioning cell. At the first analytical electrode ( $T_1$ ), the catecholamines are non-reacting but a screening reduction takes place. At the second analytical cell ( $T_2$ ) reduction of the catecholamines is accomplished.

Figure 3 shows a chromatogram of an aqueous standard containing 1 ng each of norepinephrine, epinephrine, and dopamine. 1 ng of DHBA (dihydroxybenzylamine) was added prior to the extraction as the internal standard. The extraction proceeded to a 68% recovery of DHBA. The chromatogram is complete in less than 8 minutes.

**Figure 3.**

**Chromatogram of an aqueous standard containing 1 ng each of norepinephrine, epinephrine, and dopamine.**



**Figure 4.**

**Chromatogram of a plasma sample.**

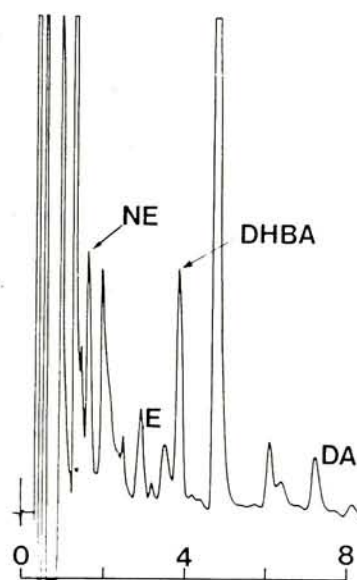


Figure 4 is a chromatogram of a plasma sample to which 1 ng of DHBA was added as the internal standard.

The mobile phase consisted of 6.9 g/L monobasic sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ), 250 mg/L heptane sulfonic acid, and 80 mg/L EDTA. The pH was adjusted to 3.6 with phosphoric acid and injected into the Model 5700 Solvent Delivery System.



# Hematoporphyrin Derivative Fluorescence: Photographic Techniques for the Localization of Malignant Tissue

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Three simple methods for the detection of malignancy by the photography of hematoporphyrin derivative (HPD) fluorescence are presented. Two methods employ a single lens reflex camera with macrolens and Kodak high-speed Ektachrome (EL 400) film. Both employ a Corning Glass #3482 filter as a barrier on the lens. In method I, a single Kodak Wratten #39 filter is placed over a fluorescent "black light" source (two General Electric F20T12BL 20-W bulbs). In method II, twin electronic flash units (5,500 BCPS unfiltered) with a double thickness of Kodak Wratten #39 filter over them provide the light of excitation. Method III employs an adapted Polaroid® SLR 680E Sun camera with two electronic flash units (5,500 BCPS, total unfiltered) rigged with a slave trigger. A Corning Glass #3482 filter is placed on the lens to act as a barrier, and two filters of Kodak Wratten #39 glass are placed on each flash unit. Photographs are taken in a darkened room.

**Key words:** tumor localization, 35-mm photography, instant photography

## INTRODUCTION

The ability to detect tumors and define their extent is of practical importance to the clinician. The porphyrins have attracted specific interest as tumor markers, since Policard observed the reddish fluorescence of endogenous porphyrins in human and animal tumors in 1924.

In 1942 Figge and Clark reported a photographic method to record this fluorescence. Peck et al [1955] used this method to demonstrate the biliary tree, lymph nodes, and neoplastic tissue in both humans and animals. Henderson et al [1980] used a similar method for the detection of hematoporphyrin diacetate. However, with the exception of a modified technique reported by Benson et al [1982], the photographic methodologies have fallen from use in favor of the development of electrooptical imaging technologies, including the use of image intensifiers [Balchum et al, 1983; Doiron et al, 1979; Hayata et al, 1982a,b, 1983; King et al, 1983; Lin et al, 1984; Profio et al, 1979, 1983; Auler and Banzer, 1942; Dougherty et al, 1985].

Advances in photographic technology have again made photographic methods practical for the detection of malignancy. Two simple methods using 35-mm photog-

raphy for the detection of tumors are described in this article.

Work in our laboratory with instant imaging of sodium fluorescein [Lanza fame et al, 1983] has provided the expertise necessary for the development of a similar methodology for the photography of HPD fluorescence. An instant photographic method for the detection and localization of tumors after injection of hematoporphyrin derivatives is also presented in this article.

## MATERIALS AND METHODS

The methods were developed and tested in both in vitro and in vivo systems. The hematoporphyrin derivatives HPD and HPD2 (Photofrin and Photofrin 2; Pho-

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tofrin Medical, Cheektowaga, NY) were prepared in pH 7.35 Dulbecco's phosphate-buffered saline (GIBCO, Grand Island, NY) at concentrations of 250, 50, 25, 10, 5, 2.5, and 1.25  $\mu\text{g/ml}$  and 750, 500, 250, 100, 75, 50, 25, and 5  $\text{ng/ml}$  and placed on 2-inch filter paper discs. All photographic exposures were made under similar conditions and with the room darkened.

The R3230AC mammary tumor was transplanted by trocar and grown to a size of 2.5 cm in 150-gm female Fisher 344 rats. The animals were given intravenous injections of HPD or HPD2 at doses of 15, 10, 5, or 3 mg/kg from 24 to 96 hours prior to photography. Each tumor was photographed in situ and after having been removed and bisected. All photographs were taken under similar conditions in a darkened room. The photographic methods are described separately.

### Method I: Black-Light Photography

A 35-mm single-lens reflex camera is fitted with a 100-mm macrolens, and the aperture is set at f4. Photographs are taken at one-third life size. An orange Corning Glass #3482 sharp cut-off filter is placed over the lens to serve as a barrier filter. The excitation light source consists of two fluorescent "black light" bulbs (General Electric F20T12BL, 20 W) over which an opaque cover with a 3-inch square window is created and covered with a blue Kodak Wratten #39 filter. This apparatus is shown in Figure 1. Kodak high-speed Ektachrome film EL400 (ASA 400) is used. All exposures are made in a darkened room with the light source 8.5 inches from the specimen. An exposure time of 30 seconds is used.

### Method II: Electronic Flash Photography

Two electronic flash units are mounted on a bracket system and angled at  $45^\circ$  relative to the subject. The total unfiltered flash output is approximately 5,500 BCPS. Each flash is fitted with a double thickness (ie, two

filters) of Kodak Wratten #39 filters. This arrangement provides a light intensity of 100 foot-candles at the specimen surface (an approximate guide number of 4). The same reflex camera, barrier filter, film, and aperture are used as described in method I. All exposures are made in a darkened room. This apparatus is demonstrated in Figure 2.

### Method III: Instant Photography

The camera system used is demonstrated in Figure 3. A Polaroid® SLR 680E Sun camera was mounted to a bracket with two electronic flash units. One of the flash units is attached to a simple slave trigger. The flash units are positioned at a  $45^\circ$  angle relative to the subject, and their combined, unfiltered output is approximately 5,500 BCPS. Each flash unit is fitted with a double thickness (ie, two filters) of Kodak Wratten #39 blue filter. This

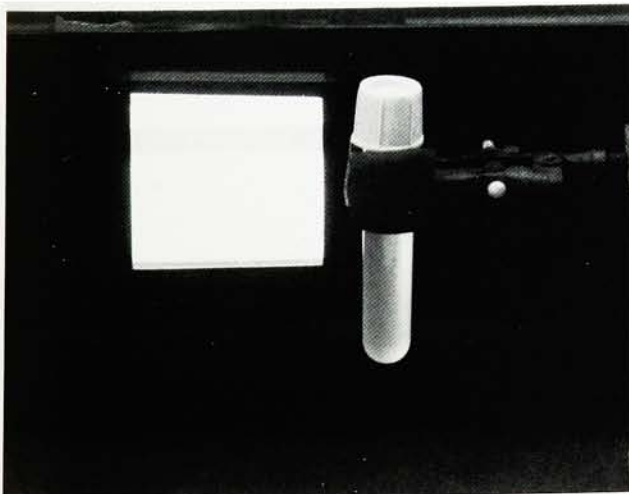


Fig. 1. Excitation light box for black-light photography.

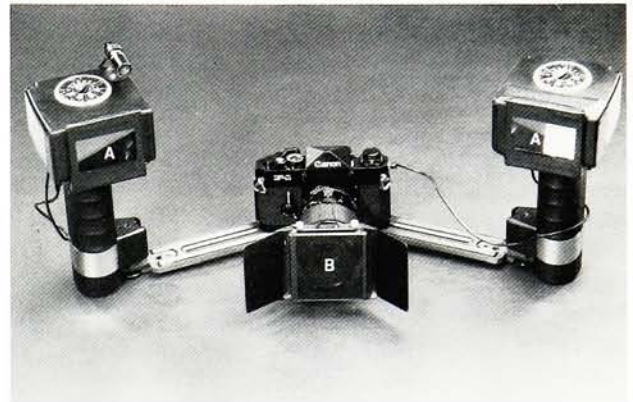


Fig. 2. Assembled flash and bracket system for fluorescence photography. A, Kodak Wratten #39 exciter filters (two placed on each flash unit as a sandwich); B, Corning Glass #3482 filter as a barrier filter.

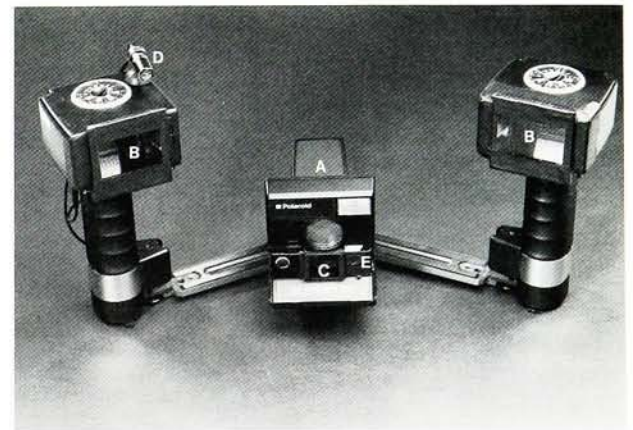


Fig. 3. Camera apparatus for method III. A, Polaroid SLR 680E camera; B, Kodak Wratten #39 filters (two thicknesses each); C, Corning #3482 glass filter; D, slave trigger; and E, black tape covering camera electronic eye.



provides 100 foot-candles of light intensity at the specimen surface and an approximate guide number of 4. The camera lens is fitted with an orange Corning Glass #3482 filter. The built-in camera flash unit is switched off, and the camera's electronic eye is covered with black tape. These maneuvers cause the aperture to open fully (f8) and the shutter to remain open for 5 seconds. Therefore, all photographs are taken in a darkened room. The photographer depresses the camera shutter and then manually triggers the electronic flash on the left, which in turn fires the second flash unit via the slave trigger. The camera is fitted with standard Polaroid High Speed Color Land Film® (ASA 600). The photographs are immediately available for diagnostic purposes if desired.

## RESULTS

The black-light and electronic flash methods produce nearly identical photographic renditions. At the appropriate concentrations, HPD (HPD2) produces a brilliant red fluorescence as compared to the background (Fig. 4). The depiction of the fluorescence phenomenon is of high contrast in contradistinction to the method of Benson et al [1982] (Fig. 5).

The *in vitro* sensitivities of method I and method II are depicted in Figure 6A and are compared to the Benson technique (Fig. 6B). The threshold of detection with both method I and method II is 50 ng/ml for both HPD and HPD2. There is a rapid decline in the intensity of the fluorescence obtained at concentrations below 1.25  $\mu\text{g/ml}$ . The Benson technique [Benson et al, 1982] is much less sensitive, with a 5  $\mu\text{g/ml}$  threshold of detection *in vitro*. There was a similar insensitivity of the Benson method in the R3230AC tumor system, with barely discernible fluorescence noted in tumors taken from animals receiving 3 mg/kg or less of HPD (HPD2) intravenously.

Both photographic methods detect tumor by demonstrating brilliant red tumor fluorescence following intravenous injection of HPD or HPD2 at all doses tested. This is demonstrated in Figure 4.

The Polaroid images produced by method III are equivalent to the images obtained with the 35-mm methods both *in vitro* and *in vivo* with the R3230AC mammary tumor models.

Tumor fluorescence as depicted by method III is demonstrated in Figure 7 and is similar to the fluorescence demonstrated in the *in vitro* studies. Each tumor fluoresces brightly after intravenous injection of HPD (HPD2). As is shown in Figure 7B, the fluorochromes are retained in the tumor for up to 96 hours following injection. Note that each sample in Figure 7B is tumor from a separate animal and that the animals received various doses of HPD (HPD2) and were biopsied from 24 to 96 hours postintravenous injection of the drug.

## DISCUSSION

The need for a simple, accurate method to detect and document malignancy cannot be underrated. The derivatives of hematoporphyrin are retained in neoplastic tissue longer than in normal tissue, permitting both tumor detection [Peck et al, 1955; Henderson et al, 1980; Benson et al, 1982; Balchum et al, 1983; Doiron et al, 1979; Hayata et al, 1982a,b, 1983; King et al, 1983; Lin et al, 1984; Profio et al, 1979, 1983; Auler and Banzer, 1942; Dougherty et al, 1985; van der Putten et al, 1983a,b; Dougherty, 1984] and selective photoradiation therapy (PRT) [Hayata et al, 1982a; Dougherty et al, 1985; Dougherty, 1984] with these drugs. The observation of tumor fluorescence when exposed to ultraviolet light [Policard, 1924; Auler and Banzer, 1942] is the foundation for the use of HPD and related compounds for tumor detection.

The excitation and emission spectra for HPD are shown in Figure 8 [Doiron et al, 1979]. These curves were generated for HPD in solution. However, *in vivo* a slight red shift is noted in both the absorption and emission spectra of HPD. By using appropriate filters, it is possible to provide a specific light of excitation, namely, light with a peak wavelength of 390–410 nm, which corresponds to the wavelengths at which HPD is most strongly excited. Both the black light and xenon arc lamp (electronic flash) are adequate sources of these wavelengths.

Since the HPD fluorescence is very weak and peaks at 630 and 690 nm, it is desirable to filter out unwanted nonfluorescent wavelengths. This serves to enhance the contrast and makes it necessary for the photographs to be taken in a darkened room. It is necessary to allow some extraneous light to reach the film to permit the definition of anatomical structures. Were this not done, the resultant image would be areas of red fluorescence on a stark black background.

The transmission curves for the #3842 Corning Glass filter and the Kodak Wratten #39 filter are shown in Figure 9. It is readily appreciated that these filters are quite suitable for the purpose of excitation of HPD and for the filtering of virtually all but the resultant fluorescence from reaching the photographic film. Preliminary work with a single Kodak Wratten #39 filter over the electronic flash (method II) produced an unacceptably high amount of background illumination. It was determined that the small window at 560 nm at a density of 3.0 was the cause (see Fig. 9B). Therefore, since densities are additive, the addition of a second filter (ie, double thickness) should reduce the amount of light transmitted from the flash without affecting the main wavelengths of excitation. This was borne out in further experiments and is the basis for the double thickness of filter used in method II and method III.

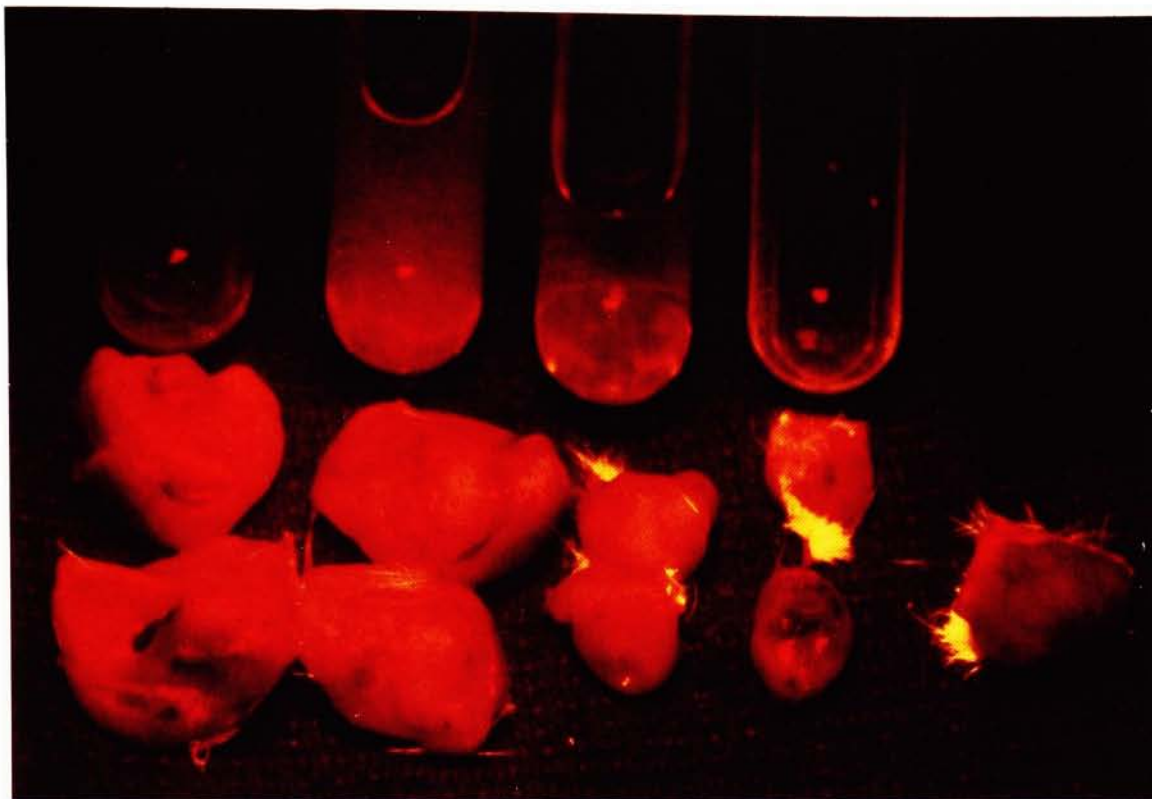


Fig. 4. Tumor fluorescence after HPD injection. Both method I and method II produce similar images.

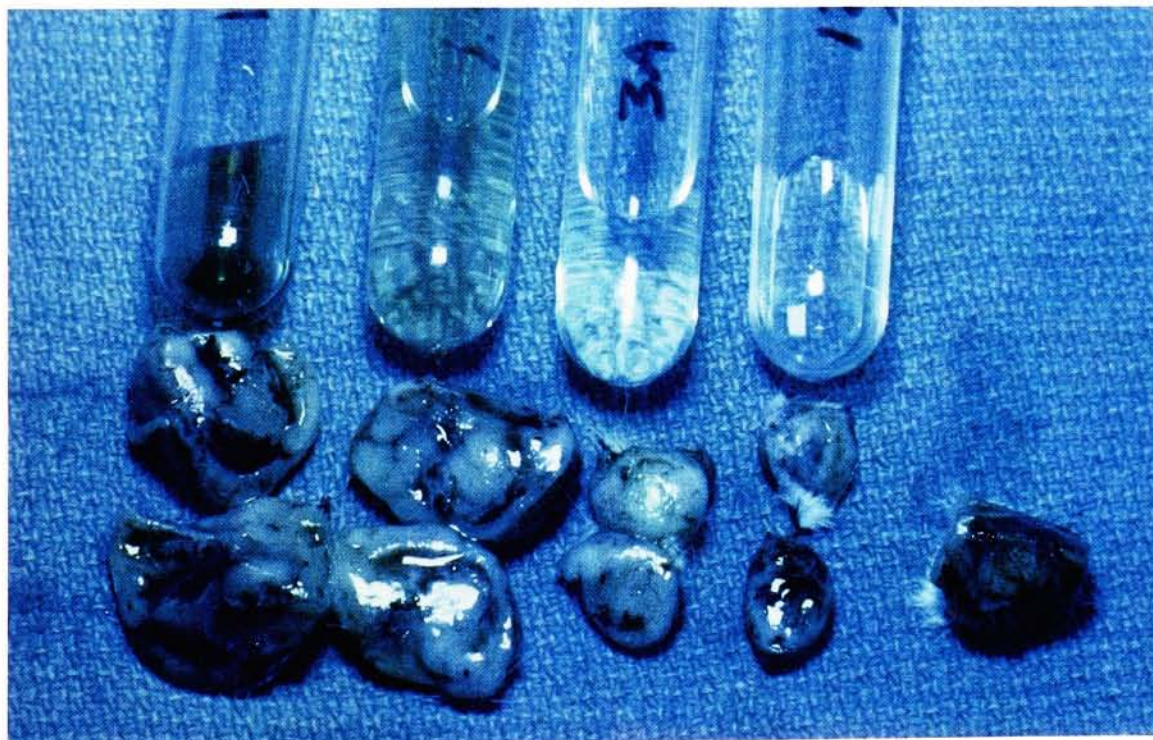


Fig. 5. Tumor fluorescence as demonstrated by the technique of Benson et al [1982]. Note the lack of contrast and sensitivity.



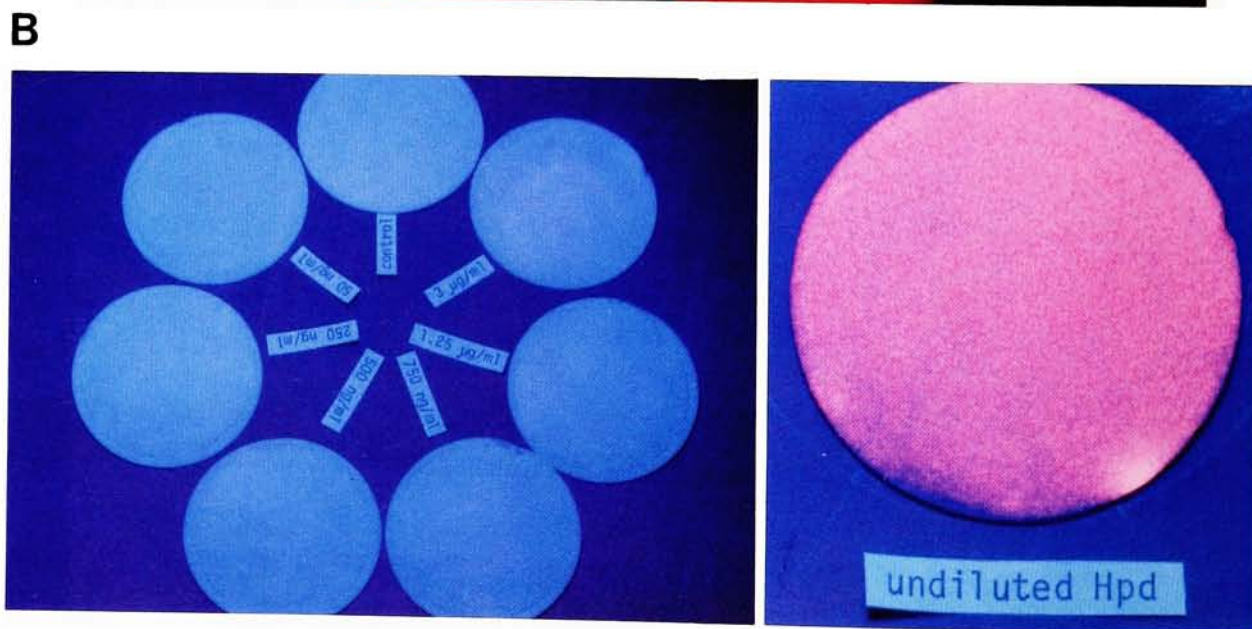
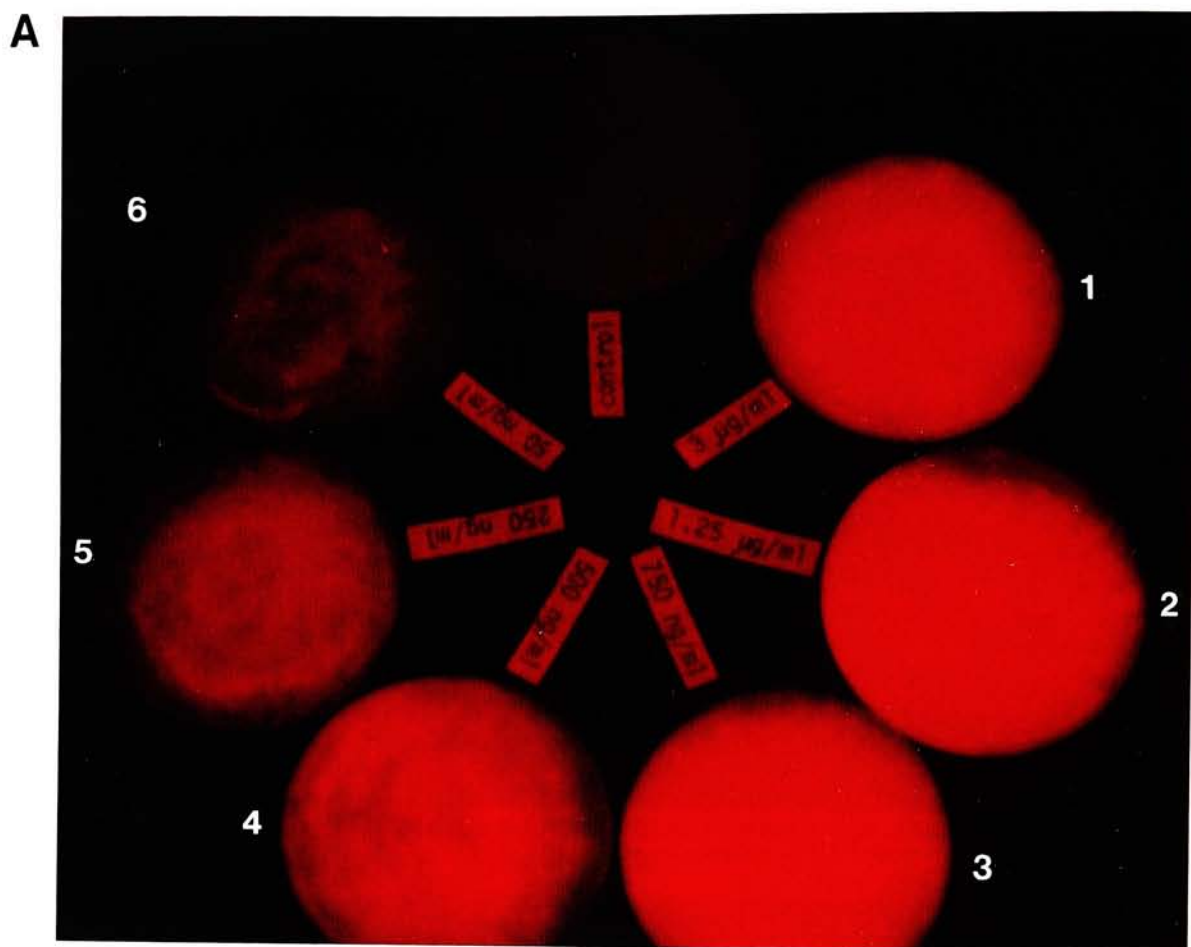


Fig. 6. In-vitro sensitivity of photography of HPD on filter paper A) Method I and method II images (note that method III produces equivalent images). 1. 3  $\mu\text{g/ml}$ ; 2. 1.25  $\mu\text{g/ml}$ ; 3. 750 ng/ml; 4. 500 ng/ml; 5. 250 ng/ml; and 6. 50 ng/ml. B) Benson technique [Benson et al, 1982].



Both method I and method II provide accurate, sensitive means of detecting HPD in malignant tissues with an in vitro threshold of 50 ng/ml and bright fluorescence at 1.25  $\mu\text{g/ml}$ . Such concentrations are well within the range of the 2–5 mg/kg doses of HPD (HPD2) used clinically.

As noted by other investigators [Dougherty et al, 1985], HPD2 causes slightly more intense tumor fluorescence than HPD, but this difference is not significant.

When photographed in solution, HPD fluoresces slightly more than HPD2.

The advantages of method II are that the system is more compact and lighter and the exposure time is much shorter. However, both methods I and II have the disadvantage of requiring film processing for the results to become apparent. Therefore, neither method is useful as a means of diagnosis, which would allow for PRT at the same sitting. It is possible, however, to document the

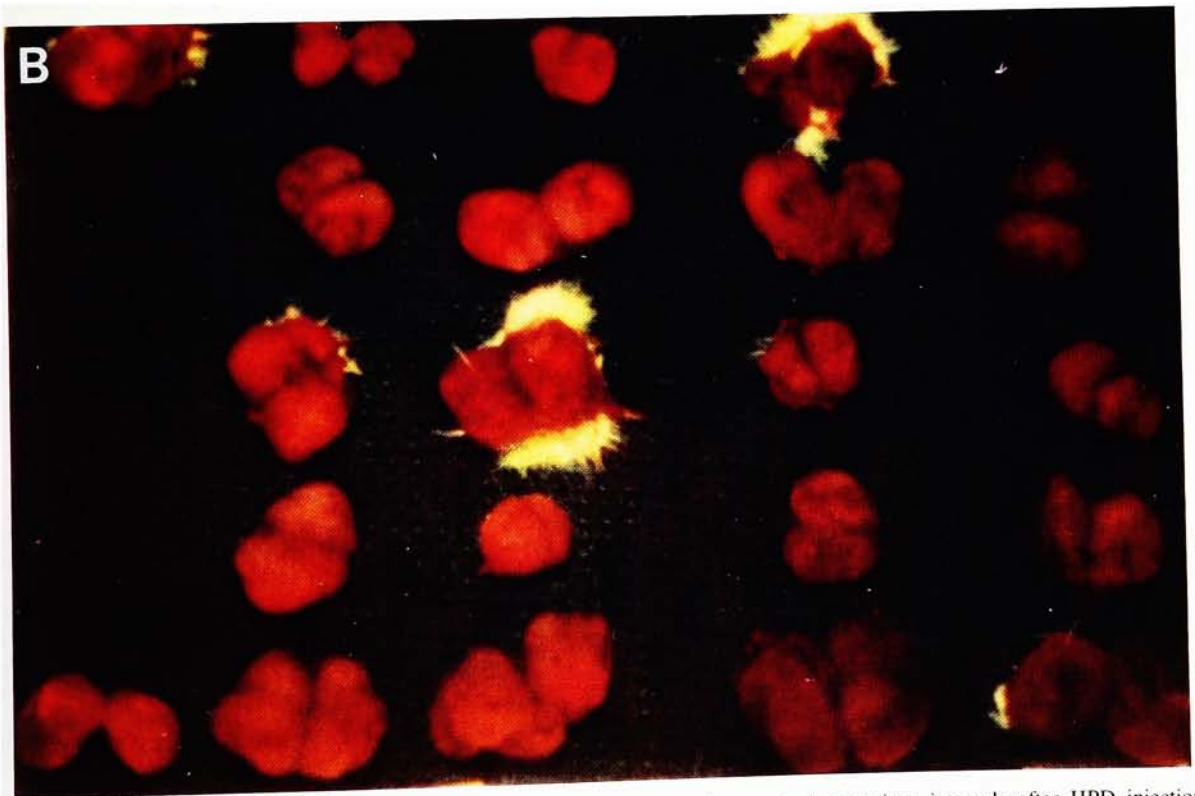
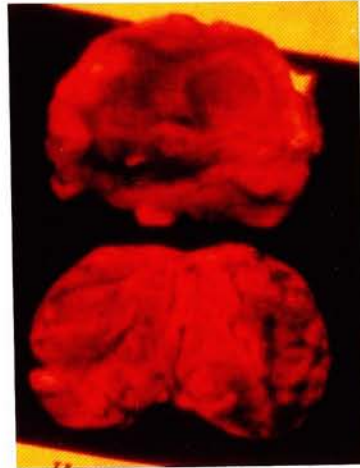
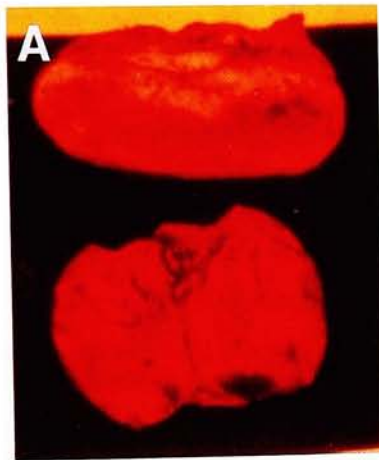


Fig. 7. In vivo photographs of fluorescence after HPD injection as depicted by method III. A) Bisected tumors photographed 48 hours after injection of 5 mg/kg HPD intravenously. B) Comparison of

tumors photographed at various intervals after HPD injection and following different injected doses of drug.

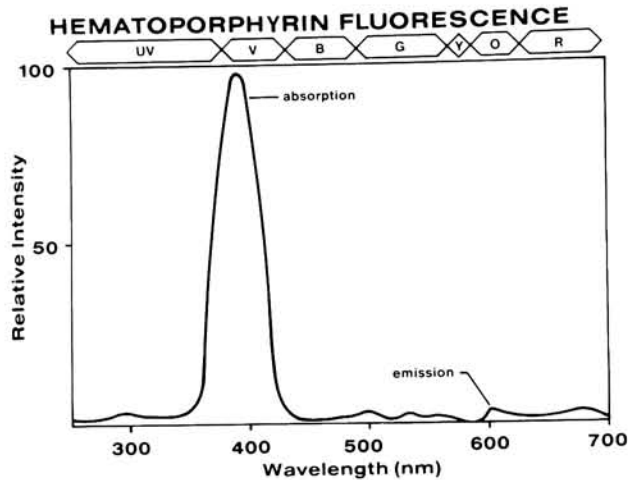


Fig. 8. Excitation and emission spectra of hematoporphyrin derivative. Adapted from Doiron DR et al [1979].

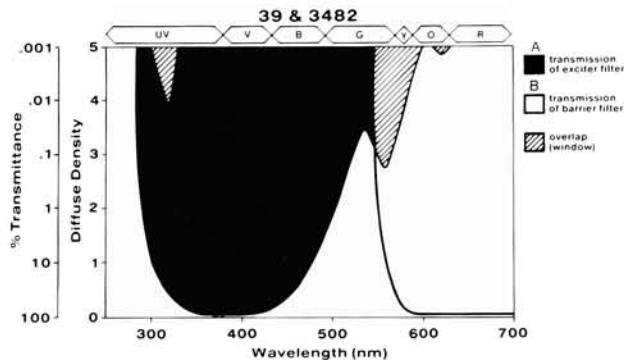


Fig. 9. Transmission curves for Kodak Wratten #39 and Corning #3482 filters. A, Kodak Wratten #39 Blue Filter; B, Corning Glass #3482 Sharp Cut-off Orange Filter.

presence of tumor and the results of therapy with these methods. Camera adaptations and the availability of laser light sources may allow the use of these methods in conjunction with endoscopic techniques.

The development of an instant photographic technique for the detection of HPD (HPD2) in tissue (method III) is of practical importance to the clinician as well as to the laboratory scientist. Such a method will allow more precise localization of neoplastic tissues and provide a basis for the delivery of photoradiation therapy (PRT) to specific areas. Method III provides immediate, permanent documentation of the local extent of disease.

van der Putten and van Gemert [1983a,b] have used a mathematical modelling theory to investigate the possible detection of subcutaneous tumors after HPD injection. Such studies indicate that it is possible to document the presence of subcutaneous tumors using this or a similar technique.

Photographic methods will be useful for monitoring the dispersion of HPD and related drugs in target tissues

following local injection or painting of lesions with these compounds. Suspension of HPD in formulations containing azone and N-methylpyrrolidone have been shown experimentally to penetrate tissues more readily than HPD alone [McCullough et al, 1983]. Photographic monitoring will allow refinement of technique and may reduce the frequency of adverse systemic effects.

Future modifications and stronger light sources may allow the endoscopist to localize and document disease in situ without the need of image intensification. Lastly, method III provides a simple, instantaneous means of documentation that the target tissue does indeed contain drug prior to the initiation of PRT.

## ACKNOWLEDGMENTS

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# Effect of Hematoporphyrin Derivative 2 on Estrogen Receptors in Experimental Mammary Tumors

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This study reports the effect of hematoporphyrin derivative 2 (HPD2) on estrogen receptors (ER) in the animal model used to develop the clinical ER assay. Fifty 200-g female Sprague-Dawley rats were given 20 mg of dimethylbenzanthracene by gastric intubation. Spontaneous mammary tumors occurred in 35 animals. Animals were anesthetized and 50% of each tumor was removed when the tumors were 2 cm in diameter. Animals were then randomized to receive 5 mg/kg (group A), 10 mg/kg (group B), or 0 mg/kg (group C) HPD2 intravenously 48 hours after biopsy. The remaining tumor was excised 48 hours post HPD2 injection. All samples were weighed, placed on ice, and frozen to  $-70^{\circ}\text{C}$ . ER assay was performed by batch run. Results (fmol/mg cytosol) were as follows: All animals ( $n=35$ ) pre HPD2  $34.2 \pm 5.4$ , post HPD2  $34.2 \pm 5.8$ ; group A: ( $n=11$ ) pre HPD2  $33.9 \pm 7.9$ , post HPD2  $37.1 \pm 7.8$ ; group B: ( $n=13$ ) pre HPD2  $29.2 \pm 3.8$ , post HPD2  $25.5 \pm 3.6$ ; group C: ( $n=11$ ) pre HPD2  $40.3 \pm 5.2$ , post HPD2  $41.5 \pm 7.9$ . HPD2 does not affect ER in this animal model. Confirmatory studies with human tumor material must be completed.

**Key words:** cancer, rodents, tumor markers, porphyrins, estrogen receptor analysis, dimethylbenzanthracene, dihematoporphyrin ether

## INTRODUCTION

Breast cancer is a common disease in the United States. Approximately 115,000 new cases are reported yearly [Cancer Statistics, 1984]. Hematoporphyrin derivative (HPD), when coupled with photodynamic therapy (PDT), effectively destroys tumor [Dahlman et al, 1983; Dougherty et al, 1978, 1979, 1981; Kinsey et al, 1983]. Results with HPDPDT in recurrent human breast carcinoma [Dougherty et al, 1979] suggest that it may someday be a useful adjunct in the treatment of primary breast cancer. However, it is imperative to determine the effect of HPD on estrogen receptor (ER) analysis prior to embarking on prospective human studies. Such information is important since ER values are key components in the planning of postoperative therapies and since it is known that factors affecting the viability of breast tumor samples may adversely affect ER analysis and accuracy. This study was undertaken to determine the effect of HPD2 on ER values.

## MATERIALS AND METHODS

The dimethylbenzanthracene (DMBA) - induced mammary tumor model was chosen because it was used to develop the clinical ER assay. Fifty 200-g female Sprague-Dawley rats were given 20 mg of DMBA by gastric intubation. Spontaneous mammary tumors oc-

curred in 35 animals at 8 to 12 weeks postintubation. All tumors occurred in the mammary ridge.

The animals were anesthetized with intraperitoneal pentobarbital and 50% of each tumor was removed when the tumors had reached 2 cm in diameter. Each sample was immediately placed on ice, weighed, and then frozen and stored at  $-70^{\circ}\text{C}$ . The animals were then randomized to receive 5 mg/kg (group A), 10 mg/kg (group B), or 0 mg/kg (group C) of HPD2 (Photofrin II; Photofrin, Inc., Raritan, NJ) intravenously 48 hours after the initial biopsy. The remainder of the tumor was excised 48 hours post HPD2 injection. Again, each sample was immediately placed on ice, weighed, and frozen at  $-70^{\circ}\text{C}$ . Histologic study of each tumor was performed to verify that it was mammary in origin.

Estrogen receptor analysis was performed by batch run radioimmunoassay technique (Rainen ER Assay; New England Nuclear, Boston, MA). All analyses were performed by a reference laboratory (Bio-Science, Inc., Great Neck, NY).

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## RESULTS

All results are reported as femtomoles per milligram (fmol/mg) cytosol and are presented in Table I. The pre HPD2 injection ER values are compared with the postinjection values for each group. Statistical analysis of the results by ANOVA was performed. There was no difference between the pretreatment ER values for each group. Similarly, the postinjection ER values were not different from the pretreatment values. These results indicate that HPD2 does not alter ER values in this animal model.

## DISCUSSION

Sixty percent of human breast carcinomas are rich in estrogen receptors [Tourville, 1984]. Approximately 60% of ER-positive patients will respond to hormonal manipulation therapy, while 90% of ER-negative patients fail to respond to treatment [Tourville, 1984; Gibson and Hilf, 1976]. It is therefore important to determine what effect any potential therapy might have on ER analysis since accurate ER information plays a key role in the planning of postoperative therapy in the breast cancer patient.

Encouraging results have been demonstrated in the treatment of advanced and recurrent human breast carcinoma [Dougherty et al, 1979]. Such therapy may be considered for adjunctive or primary treatment in human breast cancer since HPDPDT is able to destroy tumor cells locally with minimal adverse effects at distant sites and little potential of injury to adjacent structures. This therapy becomes increasingly more attractive in the growing climate of conservational breast surgery in the treatment of cancer. HPDPDT may allow the use of minimal surgical procedures with rates of cure comparable or superior to conventional modalities.

The DMBA-mammary tumor model was chosen for study because it is rich in ER. McGuire and DeLa-Garza [1973] demonstrated that these tumors contain an estradiol binding protein with similar or identical activity to human mammary carcinoma. This model served as the basis for the development of the clinical radioimmunoassay for ER.

Estrogen receptor levels are affected by sampling technique, tissue handling and storage, and factors which adversely affect cellular viability in the specimen to be analyzed [Leight et al, 1984; Meyer et al, 1983; Rosenthal, 1979; Webster et al, 1978]. Variation was minimized in this study by the sharp excision of each sample, its immediate placement on ice, and rapid freezing and storage at  $-70^{\circ}\text{C}$ . Despite this careful technique, wide variations in pre- and post-treatment values were noted in some animals. Similar results have been observed by

TABLE I. Average Estrogen Receptor (ER) Values\* (fmol/mg Cytosol)

Group	n	Pre HPD2 injection	Post HPD2 injection (48 hr)
A	11	33.9 $\pm$ 7.9	37.1 $\pm$ 7.8
B	13	29.2 $\pm$ 3.8	25.5 $\pm$ 3.6
C	11	40.3 $\pm$ 5.2	41.5 $\pm$ 7.9
Total	35	34.2 $\pm$ 5.4	34.2 $\pm$ 5.8

\*ANOVA analysis is NS for pre- and post-injection values. Differences in ER values between the groups was not significant.  $P > .05$ . HPD2, hematoporphyrin derivative 2.

Hilf and co-workers [Gibson and Hilf, 1976, 1980; Shaife and Hilf, 1978; Hilf et al, 1983]. Variability in ER values would be expected since these tumors are spontaneous and are nonhomogeneous, containing varying amounts of ER-rich tissue and fibrous stroma. Our results are in agreement with the average value of 30 fmol/mg cytosol reported for this tumor model [Gibson and Hilf, 1980].

Our results clearly demonstrate that HPD2 has no effect on ER analysis in this model. While confirmatory studies are necessary with human breast tumors, these results suggest that HPDPDT protocols are possible without fear of compromising ER analysis data.

## SUMMARY

DMBA mammary tumors were induced in 35 female Sprague-Dawley rats. Animals were randomized into groups and received 5 mg/kg, 10 mg/kg, or 0 mg/kg HPD2 intravenously. Tumors were biopsied both before and following HPD2 injection, and ER analysis was performed. There was no difference in ER activity before or after HPD2 injection. HPD2 has no effect on ER activity in this model.

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*Effect of CO<sub>2</sub> Laser Excision on Neuroblastoma.*

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Neuroblastoma is a common solid tumor in children and continues to have a poor outcome. Surgical excision is the primary treatment but can be limited by invasion around unresectable structures often leaving residual tumor. The C-1300 murine neuroblastoma is similar to the human disease and a suitable animal model. We studied the effect of CO<sub>2</sub> laser excision on the growth of residual neuroblastoma. A partial excision model was developed to simulate the clinical experience. A/J male mice were inoculated with C-1300 murine neuroblastoma. Seventy five percent excision of tumor was performed with scalpel and CO<sub>2</sub> laser (10 Watts). Residual tumor growth rates were charted in 21 mice. CO<sub>2</sub> laser excision significantly decreases the growth of residual neuroblastoma ( $p < .05$ ). Although there were no CO<sub>2</sub> laser-induced regressions of neuroblastoma, there were two observed cessations of tumor growth during the study period with CO<sub>2</sub> laser excision. We conclude that the CO<sub>2</sub> laser is a useful modality in the primary surgical treatment of neuroblastoma.



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**HELIUM CADMIUM LASER-INDUCED FLUORESCENCE OF DIHEMATOPORPHYRIN ETHER FOR THE ENDOSCOPIC DETECTION OF CANCER.**

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Laser-induced fluorescence (LIF) of dihematoporphyrin ether (DHE) is a sensitive method for the endoscopic detection of tumors. This study demonstrates helium-cadmium (HeCd) LIF of DHE in vitro in a biliary model. Detection of LIF was accomplished by direct visual inspection and both instant and 35mm photographic methods via an Olympus BF2T10 fiber-bronchoscope. DHE was placed onto filter paper discs for testing. Spectral analysis of 50ug DHE/ml 95% ETOH was obtained with a Perkin-Elmer LS-5 scanning fluorimeter. Relative fluorescence emission intensity at 630nm is as follows: 1.012 (325nm), 0.186 (406nm), 1.673 (442nm), 1.93 (514nm). A Liconix 4240NB laser (442nm, 40mW) was coupled to a Laserguide 1.5cm cylindrical diffusor (CD) or microdiverging lens (MDL) with a 19mm focusing lens and NRC F915 fiberoptic positioner. Power outputs of these systems were 1.32mW/cm<sup>2</sup> and 31.58mW/cm<sup>2</sup>, respectively at 1cm from the tip. Direct observation of LIF was possible with Laserguard Argon Safety Glasses (OD15 at 488nm, OD11 at 514.6nm) with a visual fluorescence threshold at 1.25ug DHE/ml 95% ETOH for MDL and 0.5ug DHE/ml 95% ETOH for CD. Photographic detection was accomplished by using a Corning Glass #3384 sharp cut-off barrier filter on the camera systems. Instant photographs with the Olympus SCP10 system (Polaroid 779 film, 120 sec. exposure) detects 2.5ug/ml DHE with the MDL and 1.25ug/ml DHE with the CD. 35mm photography using 15 sec. exposure time and Kodak ER135 (ASA 400) film detects DHE at a level of 1.25ug/ml DHE with the MDL. Using 1600 ASA film and a 4 sec. exposure, the system detects 0.5ug/ml DHE with the MDL and a similar concentration is detectable at an 8 sec. exposure with the CD. The HeCd laser provides a stable, inexpensive, low maintenance system for the excitation of DHE. Work is in progress with these systems to demonstrate endoscopic fluorescence of DHE in vivo.

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## PHOTOGRAPHIC DEMONSTRATION OF PENETRATION OF DIHEMATOPOR-PHYRIN ETHER PREPARATIONS INTO TUMOR SYSTEMS.

R.J. Lanzafame, M.D., D.W. Rogers, B.S., J.O. Naim, M.S., H.R. Herrera, M.D., J.R. Hinshaw, M.D., D.Phil; Department of Surgery, Rochester General Hospital, Rochester, New York. Reduction of systemic toxicity of dihematoporphyrin ether (DHE) depends on local delivery of drug to its target tissue in sufficient quantities to bind to the target while minimizing the total body dose. This paper describes the ability of DHE-admixtures to penetrate and localize in in-vitro and in-vivo tumor systems. Each system was evaluated photographically. All studies were performed using the R3230AC mammary tumor. For the in-vitro studies 24 tumors with an average weight of 2.2g were trimmed, bisected and placed in tissue culture wells containing Hanks solution. A 5mm hole was punched into the center of 24 tumor halves with a Keyes punch. Each tumor well was filled with 0.55ml of test solution: 500ug DHE in Azone: Ethanol:H<sub>2</sub>O (2:93:5) or 5ug DHE in Hanks solution. Tumors were maintained at constant temperature, removed from the cuvettes, blotted, bisected and photographed at 1/2, 2, 4, 6, 8 or 24 hours. The azone mixture resulted in DHE aggregates and tissue fixation with no penetration of DHE. The DHE/Hanks mixture resulted in 2mm penetration by 24 hours. A second study was performed by immersing bisected tumors into wells containing 500ug/DHE/Hanks or DHE:Azone: Ethanol:H<sub>2</sub>O (500ug/ml:2:48:50). The DHE/Azone/Ethanol/H<sub>2</sub>O mixture resulted in 1.5mm penetration of DHE into tumor capillaries, crevices and tissue defects. An in vivo study was performed. Test solutions were injected at 4 sites around tumors  $\geq$  1.5cm size. A concentration of 4.5mg/ml DHE dissolved in 2% Azone-Phosphate Buffered Saline (PBS), PBS or 10% lipid emulsion were injected (2ml) around the tumors. Tumors were excised, bisected post-injection and compared to negative (no DHE) and positive (15mg/kg DHE IV) controls. Each solution resulted in complete tumor penetration and fluorescence similar to the positive controls at 4 and 18 hours. The lipid emulsion had a greater tendency to remain in situ. Further studies with lipid-DHE emulsions are warranted.